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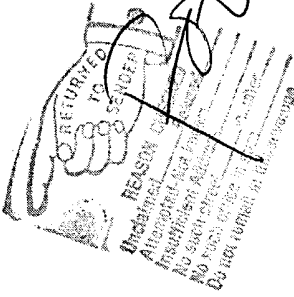
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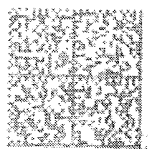
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/045,624	10/26/2001	Keith D. Allen	R-666	1008
7590 04/16/2004				
DELTAGEN, INC. 740 Bay Road Redwood City, CA 94063				
EXAMINER PARAS JR, PETER				
ART UNIT PAPER NUMBER 1632				

DATE MAILED: 04/16/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

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provide a correlation between any thyroid stimulating hormone receptor related disease/disorder and the disclosed phenotypes. The specification has provided general assertions that the claimed transgenic mice may be used to identify agents that affect a phenotype related to the mice.

As such, the asserted utility, for the transgenic mouse embraced by the claims, of screening agents that may affect a phenotype of said mouse as provided by the instant specification and encompassed by the claims, does not appear to be specific and substantial. The asserted utility does not appear specific and substantial to the skilled artisan since the evidence of record has not provided any suggestion of a correlation between any thyroid stimulating hormone receptor, the disclosed phenotypes exhibited by the transgenic mouse, and any disease or disorder. Since the evidence of record has not provided a correlation between the disclosed phenotypes and any disease or disorder, the utility of identifying agents that affect such phenotypes is not apparent. The evidence of record has not provided any other utilities for the transgenic mouse embraced by the claims that are specific, substantial, and credible.

The asserted utility of the transgenic mouse embraced by the claims is based on the expectation that disrupting the nucleotide sequence set forth in SEQ ID NO: 1 would result in a detectable phenotype in the mouse. The phenotypes observed in the transgenic mice embraced by the claims are dwarfism; hunched posture; small eyes and ears; small thymus gland; a malformed femur; small skeletal muscle; decreased fat in the subcutis; small or not visible seminal vesicles; low body weight; short body length; low organ weight (spleen, liver, kidneys, heart, thymus); low organ weight to body

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may be used to identify agents that modulate or ameliorate a phenotype associated with a disruption in SEQ ID NO: 1.

The instant specification has disclosed a transgenic mouse whose genome comprises a disruption in SEQ ID NO: 1, wherein the mouse exhibits dwarfism; hunched posture; small eyes and ears; small thymus gland; a malformed femur; small skeletal muscle; decreased fat in the subcutis; small or not visible seminal vesicles; low body weight; short body length; low organ weight (spleen, liver, kidneys, heart, thymus); low organ weight to body weight ratio (spleen, liver, kidneys); small thyroid gland with small follicles; abnormalities of the pituitary gland consisting of adenohypophysis, large and vacuolated cells, reduced chromophils, pars distalis, and chromophobe hypertrophy; dysplasia of the epiphyses of the femur, tibia and stifle joint; reduced patchy ossification of bones; reduced cellularity of bone marrow; hypoplasia with absence of cortico-medullary distinction of the thymus gland; immature kidneys with small glomeruli, lymphocytic infiltrates in the kidneys; immature testes; hypospermatogenesis; interstitial Leydig cell hyperplasia; oligospermia; lymphocytic infiltrates in the lungs; diffuse retinal fibrosis and elevated blood urea nitrogen. The claims embrace such a mouse and a method of making the mouse. The instant specification has discussed that phenotypes exhibited by such a transgenic mouse could correlate to a disease or disorder. However, the evidence of record does not provide a correlation between the disclosed phenotypes (as recited above) and any disease or disorder. Moreover, while the specification has purported that the nucleotide sequence set forth in SEQ ID NO: 1 encodes a thyroid stimulating hormone receptor, the evidence of record has failed to

Office Action Summary

Application No.

10/045,624

Applicant(s)

ALLEN, KEITH D.

Examiner

Peter Paras, Jr.

Art Unit

1632

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 30 January 2004.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 43-50 is/are pending in the application.
- 4a) Of the above claim(s) _____ is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 43-50 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

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Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
- ☐ Certified copies of the priority documents have been received.
 - ☐ Certified copies of the priority documents have been received in Application No. _____.
 - ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- ☒ Notice of References Cited (PTO-892)
- ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)
Paper No(s)/Mail Date _____
- ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____
- ☐ Notice of Informal Patent Application (PTO-152)
- ☐ Other: _____

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DETAILED ACTION

Claims 1-42 have been cancelled. New claims 43-50 have been added. Claims 43-50 are pending and are under current consideration.

Upon further consideration the following new grounds of rejection are necessary:

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claims 43-50 are rejected under 35 U.S.C. 101 because the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility.

The claims are directed to a transgenic mouse whose genome comprises a homozygous disruption in an endogenous TSH-R gene, wherein the mouse exhibits reduced growth and development, relative to a wild-type mouse. The claims are further directed to methods of making and using the same transgenic mouse.

The instant specification has contemplated that the nucleotide sequence set forth in SEQ ID NO: 1 encodes a thyroid stimulating hormone receptor. The instant specification has further contemplated that disruption of the nucleotide sequence set forth in SEQ ID NO: 1 in a mouse will produce a phenotype related to a thyroid stimulating hormone receptor. The instant specification has purported that such mice

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weight ratio (spleen, liver, kidneys); small thyroid gland with small follicles; abnormalities of the pituitary gland consisting of adenohypophysis, large and vacuolated cells, reduced chromophils, pars distalis, and chromophobe hypertrophy; dysplasia of the epiphyses of the femur, tibia and stifle joint; reduced patchy ossification of bones; reduced cellularity of bone marrow; hypoplasia with absence of cortico-medullary distinction of the thymus gland; immature kidneys with small glomeruli, lymphocytic infiltrates in the kidneys; immature testes; hypospermatogenesis; interstitial Leydig cell hyperplasia; oligospermia; lymphocytic infiltrates in the lungs; diffuse retinal fibrosis and elevated blood urea nitrogen . While the phenotypes exhibited by the claimed transgenic mouse are contemplated to be associated with a disease, the association of such phenotypes with any disease has yet to be elucidated. In fact the art suggests that phenotypes exhibited by knockout mice, are greatly influenced by the genetic background of the tested mouse. For example, Schuster-Gossler et al (Mammalian Genome, 1996, 7: 20-24) observe that a dwarfism phenotype in offspring is affected by the sex of the parent from which the transgene is inherited and also by the genetic background of the mice. See throughout the entire document. Furthermore, Schoor et al (Mechanisms of Development, 1999, 85: 73-83) discuss that phenotypes of retarded growth, peri/post natal lethality, reduced fertility, and skeletal abnormalities are influenced by the genetic background of the knockout mouse. See throughout the entire document.

Therefore, the reference suggests a need to provide independent evidence of an association of the disclosed phenotypes exhibited by the transgenic mouse embraced

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by the claims with a disease or disorder. However, neither the specification nor any art of record provides evidence of the existence of a correlation between such phenotypes and a disease or disorder, leaving the skilled artisan to speculate and investigate the uses of the transgenic mouse embraced by the claims. The specification essentially gives an invitation to experiment wherein the artisan is invited to elaborate a functional use for the transgenic mouse embraced by the claims. In light of the above, the skilled artisan would not find the asserted utility of the transgenic mouse embraced by the claims to be specific and substantial.

Claim Rejections - 35 USC § 112, 1st paragraph

The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 43-50 are also rejected under 35 U.S.C. 112, first paragraph. Specifically, since the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention.

In addition, the following rejections are maintained from the previous enablement rejection:

The aspect of the previous enablement rejection relating to phenotypes of transgenic knockout mice is maintained for the reasons of record advanced on pages 8-10 of the Office action mailed on 9/26/03.

Applicant's arguments filed 1/30/04 have been fully considered but they are not persuasive. Applicants contend that the pending claims now recite a homozygous transgenic mouse exhibiting a phenotype of reduced growth and development resulting from disruption of a TSH-R are fully enabled and described by the instant specification.

In response, the Examiner asserts the pending claims are not fully enabled for phenotypes resulting from disruption of any TSH-R gene. Rather, the phenotypes recited in the claims result from disruption of the nucleotide sequence set forth in SEQ ID NO: 1. It is further maintained that phenotypes resulting from disruption of a gene are unpredictable as set forth in the previous Office action. See Moreadith and Moens on pages 8-9 of the Office action mailed on 9/26/03. Also see Leonard et al (Immunological Reviews, 1995, pages 97-114) who discuss that inactivation of the gene encoding cytokine receptor γ chain in transgenic mice results in a phenotype different from that expected. The recited phenotypes are specific only for disruption of the nucleotide sequence set forth in SEQ ID NO: 1 and not for disruption of any TSH-R gene. Also, the breadth of the phenotypes recited in claim 43 is not fully enabled by the guidance provided by the instant specification. The claim recites phenotypes of reduced growth and development. However, the breadth of such encompasses any growth or developmental abnormality while the specification has only disclosed specific growth or developmental abnormalities resulting from disruption of the nucleotide sequence of SEQ ID NO: 1. As discussed above, phenotypes resulting from disruption of a gene are unpredictable.

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Accordingly, the aspect of the previous enablement rejection relating to unpredictability of phenotypes resulting from disruption of a gene is maintained for the reasons of record.

The aspect of the previous rejection relating to embryonic stem cells has been withdrawn in light of the newly presented claims.

Conclusion

No claim is allowed.

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Any inquiry concerning this communication or earlier communications from the examiner(s) should be directed to Peter Paras, Jr., whose telephone number is (571) 272-0732. The examiner can normally be reached Monday-Friday from 8:30 to 4:30 (Eastern time).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amy Nelson, can be reached at 571-272-0804. Papers related to this application may be submitted by facsimile transmission. Papers should be faxed via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Official Fax Center number is (703) 872-9306.

Inquiries of a general nature or relating to the status of the application should be directed to Dianiece Jacobs whose telephone number is (571) 272-0532.

Peter Paras, Jr.

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**PETER PARAS, JR.
PRIMARY EXAMINER**

A handwritten signature in cursive script that reads "Pete Paras".

Notice of References Cited	Application/Control No. 10/045,624	Applicant(s)/Patent Under Reexamination ALLEN, KEITH D.	
	Examiner Peter Paras, Jr.	Art Unit 1632	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
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	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Leonard et al. Immunological Reviews, 1995, 148: 97-114.
	V	Schoor et al. Mechanisms of Development, 1999, 85: 73-83.
	W	Schuster-Gossler et al. Mammalian Genome, 1996, 7: 20-24.
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Role of the Common Cytokine Receptor γ Chain in Cytokine Signaling and Lymphoid Development

WARREN J. LEONARD¹, ELIZABETH W. SHORES² & PAUL E. LOVE

INTRODUCTION

X-linked severe combined immunodeficiency (XSCID) is an inherited disease in which patients exhibit profoundly diminished cell-mediated and humoral immunity. This disease results from mutations in the common cytokine receptor γ chain, γ_c . In order to understand more about the role of γ_c in lymphoid development, we have analyzed mice in which the γ_c gene was specifically inactivated by homologous recombination. These mice exhibit a wide range of interesting immunological abnormalities, some shared by humans with XSCID and some that are different. The differences are indicative of variations in lymphoid development between humans and mice, and potentially will allow new insights into the roles of γ_c -dependent cytokines in both species. Moreover, the mutant (γ_c^{-Y}) mice provide a valuable animal model of γ_c deficiency and therefore represent potential targets for reconstitution by gene therapeutic approaches.

X-LINKED SEVERE COMBINED IMMUNODEFICIENCY: IDENTIFICATION OF THE MOLECULAR DEFECT

Severe combined immunodeficiency diseases represent a spectrum of disorders (reviewed in Leonard et al. 1994, Leonard, in press), with XSCID accounting for ap-

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Correspondence to: Dr. Warren J. Leonard, Bldg. 10, Rm. 7N244, NHLBI, Bethesda, MD 20892-1674

proximately half of all cases of SCID (Conley 1992). Affected males exhibit profoundly diminished numbers of T cells, explaining the lack of cell-mediated immunity. B-cell numbers are normal or even increased, but are nonfunctional. The lack of B-cell function has been assumed to result in part from a lack of T-cell help since the addition of normal T cells *in vitro* can promote at least some B-cell responses (Conley 1992). However, an intrinsic B-cell defect is also indicated by the non-random X-inactivation patterns observed in the mature B cells of XSCID carrier females (Conley 1992). XSCID patients also lack natural killer (NK) cells. In contrast to these lymphoid abnormalities, other blood cells are represented in normal numbers and exhibit normal function.

The discovery of the molecular defect in XSCID came not from studies targeted to that end, but rather from basic studies on the IL-2 receptor. This receptor is known to contain at least three subunits, the α (Leonard et al. 1982, Leonard et al. 1984, Nikaido et al. 1984, Cosman et al. 1984), β (Sharon et al. 1986, Tsudo et al. 1986, Teshigawara et al. 1987, Hatakeyama et al. 1989) and γ (Takeshita et al. 1992) chains. These three chains together are responsible for forming three classes of IL-2 receptors. Whereas intermediate affinity receptors ($\beta + \gamma$ chains) are expressed on resting lymphocytes, high affinity ($\alpha + \beta + \gamma$ chains) and low affinity (α chains without β or γ) receptors are found on activated T cells (Leonard et al. 1994, Taniguchi 1995, Leonard, in press). The high and intermediate affinity receptors are capable of transducing IL-2 signals, whereas low affinity receptors cannot. Corresponding to this observation, it is interesting that both β and γ chains (the shared components of high and intermediate affinity receptors) are members of the cytokine receptor superfamily (Bazan 1990), whereas α is not. Experiments using chimeric receptor constructs indicate that heterodimerization of the cytoplasmic domains of β and γ is necessary and sufficient for signaling (Nakamura et al. 1994, Nelson et al. 1994).

Analysis of the γ chain gene revealed that it was located on the X chromosome at position Xq13 (Noguchi et al. 1993c) previously established to be the locus of XSCID (the SCIDX1 locus) (de Saint Basile et al. 1987). Sequencing of DNA prepared from EBV infected cell lines from XSCID patients subsequently established that the γ chain gene was defective in XSCID (Noguchi et al. 1993c). A large number of patients with XSCID have now been studied (reviewed in Leonard, in press). The range of identified mutations include a number of premature stop codons, insertions, deletions and splice junction defects of such a nature that even if the γ chain mRNA and protein product were stably produced, functional alterations would be expected in the protein. In addition, a number of point mutations have been found that result in single amino acid changes (Puck et al. 1993, DiSanto et al. 1994a, 1994b, Ishii et al. 1994, Russell et al. 1994, Clark et al. 1995). In some cases, amino acid alterations in the extracellular domain have been demonstrated to inhibit IL-2 binding, whereas mutations in the cytoplasmic domain interfere with signal transduction. Thus, a number of naturally occurring mutations have helped to clarify the functional domains of the γ chain protein.

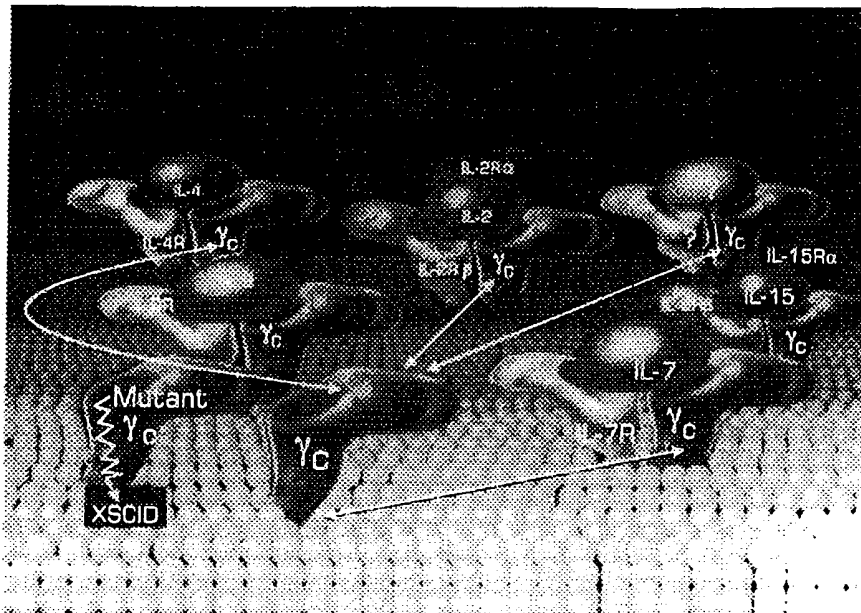


Figure 1. Schematic of the common cytokine receptor γ chain (γ_c) as a component of the IL-2, IL-4, IL-7, IL-9 and IL-15 receptors. In males, XSCID results from mutations in γ_c .

A COMMON CYTOKINE RECEPTOR SUBUNIT

One of the puzzling features of the discovery that defects in γ chain caused XSCID in humans was that, in contrast to the profound block in T-cell development observed in XSCID patients, mice (Schorle et al. 1991) and humans (Pahwa et al. 1989, Weinberg & Parkman 1990) lacking IL-2 expression exhibit normal T-cell development. In other words, mutations in a component of the IL-2 receptor yielded defects more severe than those found associated with IL-2 deficiency. This observation led to the hypothesis that the γ chain was a component of other cytokine receptors as well (Noguchi et al. 1993), consistent with the precedents that the hematopoietic cytokines IL-3, IL-5 and GM-CSF all share a common β chain, β_c (Miyajima et al. 1992), and that another set of cytokines all share the IL-6 receptor signal transducer gp130 (Taga & Kishimoto 1995). Although the γ chain was initially hypothesized (see Leonard et al. 1994 for the rationale underlying this hypothesis) and confirmed (Kondo et al. 1993, Russell et al. 1993, Noguchi et al. 1993b, Kondo et al. 1994) to be a component of the IL-4 and IL-7 receptors, it is now also known to be a component of the IL-9 (Russell et al. 1994, Kimura et al. 1995) and IL-15 (Giri et al. 1994) receptors as well. Since the γ chain is shared by multiple cytokine receptors (Figure 1), it is now denoted as the common cytokine receptor γ chain, γ_c , in keeping with the nomenclature adopted for the hematopoi-

etic cytokines (Miyajima et al. 1992). The overall clinical features of XSCID can ostensibly be accounted for by the simultaneous inactivation of five cytokine systems in patients with this disease. In all likelihood, inactivation of IL-7 signaling is primarily responsible for the lack of T-cell development in this disease (Leonard 1994, Leonard, in press), particularly in view of the phenotype of IL-7 deficient mice (von Freeden-Jeffry et al. 1995).

JANUS FAMILY TYROSINE KINASES JAK1 AND JAK3

As noted above, heterodimerization of IL-2R β and γ_c is sufficient to trigger cell proliferation. To understand this phenomenon, it was necessary to identify the essential signaling molecule(s) that associate with each chain. It was striking that stimulation of activated T cells or NK cells by IL-2 rapidly induced the tyrosine phosphorylation of Jak1 and Jak3, two different Janus family tyrosine kinases (Johnston et al. 1994, Witthuhn et al. 1994). An analysis of protein-protein interactions revealed that Jak1 associates with IL2R β and Jak3 associates with γ_c (Boussiotis et al. 1994, Russell et al. 1994, Miyazaki et al. 1994). Moreover, Jak3 can also interact with IL-2R β following IL-2 stimulation (Russell et al. 1994). The importance of the γ_c -Jak3 interaction was established by the identification of a family pedigree with an X-linked immunodeficiency in which there was a single amino acid change in γ_c (Leu 271 mutated to Gln) (Schmalstieg et al. 1995, Russell et al. 1994). This mutation was shown to greatly diminish the ability of γ_c to interact with Jak3 (Russell et al. 1994). A particularly exciting aspect of this finding was that affected individuals in this family have a moderate rather than severe form of immunodeficiency; therefore, the partial loss of Jak3 association corresponded to the partial loss of immune function. These data suggest that XSCID may result from mutations of γ_c that directly or indirectly prevent the activation of Jak3 (i.e. either by inhibiting cytokine binding or by preventing γ_c -Jak3 association). Moreover, some autosomal recessive cases of SCID that are clinically and immunologically indistinguishable from XSCID have been found to result from mutations in Jak3 (Macchi et al. 1995, Russell et al. 1995).

DEVELOPMENT OF A MURINE MODEL OF γ_c -DEFICIENCY

Given the profound abnormalities in humans with XSCID, we wished to develop a murine model of γ_c deficiency on the assumption that such animals would (1) allow more detailed analysis of the role of γ_c in lymphoid development, (2) clarify the similarities and differences in γ_c function in humans and mice, (3) serve as a murine model for potential reconstitution of γ_c in distinct lineages, (4) allow structure-function relationships for γ_c , and (5) serve as an animal model of XSCID, thereby facilitating the evaluation of gene therapeutic approaches. Successful gene therapy of mice using already established γ_c -expressing viruses (Qazilbash et al. 1995) could increase the rationale for progressing to gene therapy in humans. A

naturally occurring form of XSCID in dogs (Henthorn et al. 1994, Somberg et al. 1994) may also be valuable in this regard.

CHARACTERIZATION OF THE MURINE γ_c GENE, PREPARATION OF A TARGETING VECTOR, AND GENERATION OF γ_c -DEFICIENT MICE

The murine γ_c gene, like the human gene, is located on the X chromosome; in mice, γ_c maps between *Zfx* and *Plp* (Cao et al. 1993). The murine cDNA has 80% amino acid identity to human γ_c (Cao et al. 1993). The entire gene was cloned and sequenced (Cao et al. 1995). As expected, like the human γ_c gene (Noguchi et al. 1993a), the murine gene is divided into eight exons. A sequence-replacement targeting vector was prepared by standard methodology (Tybulewicz et al. 1991), the vector was transfected into embryonic stem (ES) cells, and homologous recombination achieved (Cao et al. 1995). Because γ_c is located on the X chromosome and the ES cells are of male lineage, a single recombination event was sufficient to create a γ_c -null cell line. The ES cells were injected into C57BL/6 blastocysts, chimeric mice were generated, and germline transmission was achieved. Mating of heterozygous ($\gamma_c^{+/-}$) females with wild-type males or of heterozygous females with $\gamma_c^{-/Y}$ males yielded progeny with the expected ratios based on Mendelian genetics (Cao et al. 1995). In an independent study, $\gamma_c^{-/Y}$ mice were also generated by DiSanto et al. (1995) using a cre-loxP recombination approach.

GROSS AND MICROSCOPIC ANALYSIS OF $\gamma_c^{-/Y}$ MICE

$\gamma_c^{-/-}$ females, $\gamma_c^{+/-}$ females, and $\gamma_c^{-/Y}$ males were indistinguishable in gross appearance from wild-type littermates. Housed under SPF conditions, γ_c -deficient mice that have been followed for up to 7 months appear healthy (our unpublished observations). However, three mice have developed a wasting syndrome characterized by runted appearance, matted hair, and failure to thrive (see below).

At necropsy, heterozygous females were indistinguishable from wild-type males and females, consistent with the normal phenotype of human XSCID carrier females. In XSCID carrier females, strictly nonrandom X-inactivation patterns are observed in T cells, NK cells, and mature B cells (reviewed in Conley 1992, Puck 1993, Leonard et al. 1994), indicating that only those cells in which the mutant X was inactivated could mature and terminally differentiate. Thus, the levels of γ_c in cells of carrier females are normal, equivalent to individuals who have wild-type γ_c at both alleles. Presumably, the same conditions operate in $\gamma_c^{+/-}$ female mice.

The $\gamma_c^{-/Y}$ males examined were all abnormal, but the phenotype differed to some extent, depending on the age of the mice. These data are largely from Cao et al. (1995) and are summarized in Table I. The youngest mice analyzed (3-week-old mice) had small thymuses and small spleens, but otherwise had grossly

TABLE I

Properties of γ_c knockout mice

1. Small hypoplastic thymuses with increased CD4:CD8 ratio.
2. Small hypoplastic spleens at 3 weeks of age but then increasing in cellularity and size. Increased CD4:CD8 ratio.
3. Greatly diminished conventional B cells in bone marrow and spleen but detectable peritoneal B-1 cells.
4. Absent peripheral lymph nodes. Inactive follicles in mesenteric nodes.
5. No gut-associated lymphoid tissue.
6. No $\gamma\delta$ cells in skin, gut, or thymus.
7. No NK cells.
8. Striking γ_c -independent proliferation in thymus.
9. Readily detectable IgM levels; other Ig levels severely diminished.

normal chest and abdominal cavity organs. The slightly older animals that have been examined, ranging in age from 4 to 9 weeks of age, also had small thymuses, but surprisingly, the spleens in these animals were increased in size. Microscopic examination of $\gamma_c^{-/-}$ thymuses revealed marked lymphoid hypoplasia with an indistinct corticomedullary junction (Cao et al. 1995). Hassall's corpuscles and a cortical rim of lymphocytes could be identified and basic thymic architecture was retained, in contrast to what is seen in SCID/NCR mice. At 3 weeks of age, the spleens were remarkable for their diminished white pulp and lymphocyte hypoplasia. In contrast, the red pulp was indistinguishable from that of normal littermates. However, with increasing age, the spleens increased in size and cellularity.

Although lymph nodes were not grossly observed in $\gamma_c^{-/-}$ mice, at necropsy, a mesenteric node was identified in a $\gamma_c^{-/-}$ mouse (Cao et al. 1995). The node was much smaller than mesenteric nodes in normal sibling mice; moreover, the single lymphoid follicle identified lacked a germinal center.

Remarkably, no gut-associated lymphoid tissue could be identified in either the small or large intestine of $\gamma_c^{-/-}$ mice. Significantly, all of the $\gamma_c^{-/-}$ and $\gamma_c^{-/-}$ mice necropsied to date (n=13) exhibited enlarged spleens and thickening of the large bowel. Histological examination of the bowel revealed proliferative typhlitis (inflammation of the cecum) and colitis, with an increase in crypt depth. Intestinal intraepithelial lymphocytes (IELs) were notably absent, but abundant mononuclear infiltrates were observed in the lamina propria. Inflammatory bowel disease (IBD) has also been reported in several other immunodeficient lines of mice generated by gene targeting, including mice lacking IL-2 (Sadlack et al. 1993), IL-10 (Kuhn et al. 1993), MHC class II, or the T cell receptor α or β chains (Mombaerts et al. 1993). Although none of these mice exhibit disease identical to human IBD (ulcerative colitis or Crohn's disease), many characteristic features are present including crypt abscesses, mucosal ulcerations and granulomas. Absence

of these features in γ_c -deficient mice could reflect a different etiology of IBD; however, further analysis is required, particularly with older mice. Interestingly, large numbers of *Helicobacter hepaticus* were observed in the crypts and lumens of the cecum and colon of $\gamma_c^{-/-}$ males (Cao et al. 1995). While these findings are consistent with speculation that an immune response to gut bacterial flora (either antigen specific or mitogen driven) may contribute to the development of IBD in gene-targeted (Strober & Ehrhardt 1993, MacDonald 1994) and SCID (Russell et al. 1995) mice, a causal relationship between *H. hepaticus* and the inflammatory changes in the $\gamma_c^{-/-}$ mice has not been established.

PHENOTYPIC ANALYSIS OF T AND B CELLS IN $\gamma_c^{-/-}$ MICE

Although thymocytes were greatly diminished in total numbers, flow cytometric analysis of thymocytes revealed that CD4⁻CD8⁻ double negative (DN), CD4⁺CD8⁻ and CD4⁺CD8⁺ single positive (SP), and CD4⁺CD8⁺ double positive (DP) cells were all represented (Cao et al. 1995). The $\gamma_c^{-/-}$ thymocytes appeared to include a relatively high proportion of mature cells, with a relative increase in the number of cells bright for TCR β , CD3 ϵ , and CD5, and a corresponding decrease in the number of cells expressing high levels of HSA. Interestingly, there was a significant increase in the CD4⁺CD8⁻:CD4⁺CD8⁺ ratio in the thymus. Although a role for specific cytokines and their receptors has not been established in positive selection, the increased percentage of CD4⁺ thymocytes suggests this possibility. Splenocytes also exhibited an increase in the CD4⁺CD8⁻:CD4⁺CD8⁺ ratio (Cao et al. 1995), consistent with the skewing observed in the thymus. These appeared to be conventional CD4⁺ T cells and not the population of class I MHC educated CD4⁺ T cells (Bendelac et al. 1994, Coles & Raulet 1994, Lantz & Bendelac 1994) since few cells were TCR V β 8 positive and they did not stain with antibody to NK1.1 (Cao et al. 1995). In fact, no NK1.1⁺ cells were identified, consistent with the lack of natural killer cytolytic activity in $\gamma_c^{-/-}$ mice (Cao et al. 1995). In contrast, granulocytes (Gr1⁺ cells) and monocytes/macrophages (Mac1⁺ cells) were increased.

Analysis of B cells in both spleen and bone marrow by staining for B220 and μ revealed a significant decrease in the number of conventional B cells. There was also a decrease in the number of B220⁺ CD43⁻ cells in the bone marrow of γ_c -deficient mice (unpublished data; DiSanto et al. 1995), consistent with a requirement for IL-7R in B cell development (Peschon et al. 1994). In contrast, "self replenishing" peritoneal B-1 cells (Kantor & Herzenberg 1993) were less affected. Both CD5⁺ and CD5⁻ B-1 cells were identified in γ_c -deficient mice. The presence of these cells may explain the detectable levels of serum IgM expressed in the $\gamma_c^{-/-}$ mice; the other classes of immunoglobulin were greatly diminished (Cao et al. 1995). This finding was expected for IgG1 and IgE, given that in the absence of γ_c , IL-4 signaling should be defective, and is also consistent with the phenotype of IL-4^{-/-} mice (Kuhn et al. 1991).

As mentioned above, humans with XSCID generally lack T cells, so it was surprising that significant numbers of T cells were found in $\gamma_c^{-/Y}$ mice (Cao et al. 1995). The T cells appeared to be strictly $\alpha\beta$ TCR⁺ as $\gamma\delta$ T cells were not identified in the thymus or spleen. Moreover, examination of the skin revealed that although Langerhans cells were normal in number and distribution, $\gamma\delta$ TCR⁺ dendritic epidermal T cells were absent. iIELs were also absent, and specific staining for $\gamma\delta$ TCR⁺ cells in the intestine was negative. Thus, it appears that $\gamma_c^{-/Y}$ mice exhibit a total absence of all populations of $\gamma\delta$ T cells, whereas at least a subset of $\alpha\beta$ T cells are able to mature and populate the peripheral lymphoid tissue (Cao et al. 1995). The lack of $\gamma\delta$ T cells could be responsible in part for the relative paucity of $\alpha\beta$ TCR⁺ thymocytes and T cells in $\gamma_c^{-/Y}$ mice as previous studies have suggested that $\gamma\delta$ TCR⁺ thymocytes can contribute to the development of $\alpha\beta$ T cells (Shores et al. 1990, Ferrick et al. 1990, Iwashima et al. 1991).

The presence of T cells in the spleen but almost complete absence of lymph nodes in $\gamma_c^{-/Y}$ mice was unexpected. One possible explanation for these findings is that γ_c -dependent cytokine signals might participate in T cell homing to lymph nodes. Alternatively, in the absence of appropriately activated lymphocytes (and the factors they secrete), endothelial cells may not be induced to a state capable of supporting lymphocyte homing. While these remain intriguing possibilities, additional studies are required to clarify the basis for the decreased lymph node mass.

One of the most interesting findings in $\gamma_c^{-/Y}$ mice was the increase in numbers of CD4⁺ T cells, particularly with advancing age. This is unlikely to be a result of expansion as peripheral T cells from these mice failed to proliferate in culture (Cao et al. 1995). The marked accumulation of T cells in $\gamma_c^{-/Y}$ mice with advancing age is also consistent with the proposed role of γ_c -mediated signals in peripheral negative selection. Whereas T cells stimulated through the TCR in the absence of co-stimulation usually enter a state of anergy, it has been shown that induction of anergy could be prevented if concomitant signals were delivered through γ_c (Boussiotis et al. 1994). Results from another study suggest that IL-2 is important for the induction of cell death. High doses of antigen in combination with IL-2 have been shown to induce cell death both in antigen-specific cell lines and in a transgenic model of EAE (Critchfield et al. 1994). Hence, it is possible that the T cells that accumulate in $\gamma_c^{-/Y}$ mice represent "anergized" T cells with potentially autoreactive TCR specificities.

COMPARISON OF T- AND B-CELL ABNORMALITIES IN HUMANS WITH XSCID AND $\gamma_c^{-/Y}$ MICE

As noted above, in human XSCID, T cells are absent or profoundly diminished in numbers. In contrast, in $\gamma_c^{-/Y}$ mice, though diminished, T cells are present and with

time can increase to essentially normal numbers in the spleen (Cao et al. 1995). Although peripheral T cells from $\gamma_c^{-/-}$ mice did not respond to stimulation with either concanavalin A or to PMA + IL-4, stimuli strictly dependent on γ_c , there was modest proliferation to anti-CD3 + anti-CD28 and to PMA + ionomycin (Cao et al. 1995). Splenocyte proliferation was approximately 10 to 20% of wild-type control levels with PMA + ionomycin and approximately 5% of control levels with anti-CD3 + anti-CD28 (Cao et al. 1995). However, quite unexpectedly, thymocytes from γ_c -deficient mice proliferated as well as control thymocytes in response to stimulation with anti-CD3 + anti-CD28 (Figure 2; Cao et al. 1995).

These findings were surprising for at least two reasons. First, they indicated that T-cell development could proceed in the absence of γ_c in mice. Second, they revealed that T cells, but more strikingly thymocytes, can be stimulated to proliferate independently of γ_c -mediated signals. Both of these findings could be explained by the actions of cytokine(s) whose receptors do not contain γ_c . Although it is unclear which cytokine(s) might be responsible, it may be relevant that IL-7R $^{-/-}$ mice exhibit a more severe phenotype (Peschon et al. 1994) than do $\gamma_c^{-/-}$ mice (Cao et al. 1995). This finding initially seems remarkable given that the absence of γ_c should inactivate IL-2, IL-4, IL-7, IL-9, and IL-15 signaling. Thus, one might assume that IL-7R $^{-/-}$ mice would respond to all cytokines except IL-7. However, this may not in fact be the case. Loss of IL-7R would also inactivate signaling in response to another cytokine, thymic stromal derived lymphopoietin (TSLP) (Peschon et al. 1994). The TSLP receptor contains IL-7R but it has not been determined whether it contains γ_c . Thus, we speculate that TSLP action contributes to T-cell development in the $\gamma_c^{-/-}$ mice (Cao et al. 1995). Since no human homologue of TSLP has been identified, it is conceivable that humans with XSCID lack this cytokine to compensate for the loss of IL-7 action and therefore exhibit more severe defects than do $\gamma_c^{-/-}$ mice.

Regarding B-cell development, it is notable that IL-7R $^{-/-}$ mice, IL-7R $^{-/-}$ mice and mice treated with anti-IL-7 antibodies both exhibit a block in B-cell development (von Freeden-Jeffry et al. 1995, Peschon et al. 1994, Grabstein et al. 1993). Thus, in $\gamma_c^{-/-}$ mice, it seems likely that the block in development of conventional B cells (Cao et al. 1995) results from inactivation of IL-7 signaling, consistent with the action of IL-7 as a pre-B-cell growth factor. These findings also support the contention that B-1 peritoneal B cells, whose development is less affected in $\gamma_c^{-/-}$ mice, are derived from a separate lineage from conventional B cells (Kantor & Herzenberg 1993). It is striking that in human XSCID, B cells are normal or even increased in number, albeit nonfunctional. This observation suggests either that IL-7 is a major pre-B cell growth factor in mice, but not in humans, or that alternative γ_c -independent pathway(s) exist to support B-cell development in humans. Although this hypothesis is attractive, it should be emphasized that other explanations could exist to explain the observed differences between XSCID patients and $\gamma_c^{-/-}$ mice. For example, the degree to which maternal factors (eg., cytokines) influence fetal development may not be identical in both species.

THYMOCYTES FROM γ_C -KNOCKOUT MICE RESPOND TO γ_C -INDEPENDENT BUT NOT γ_C -DEPENDENT STIMULI

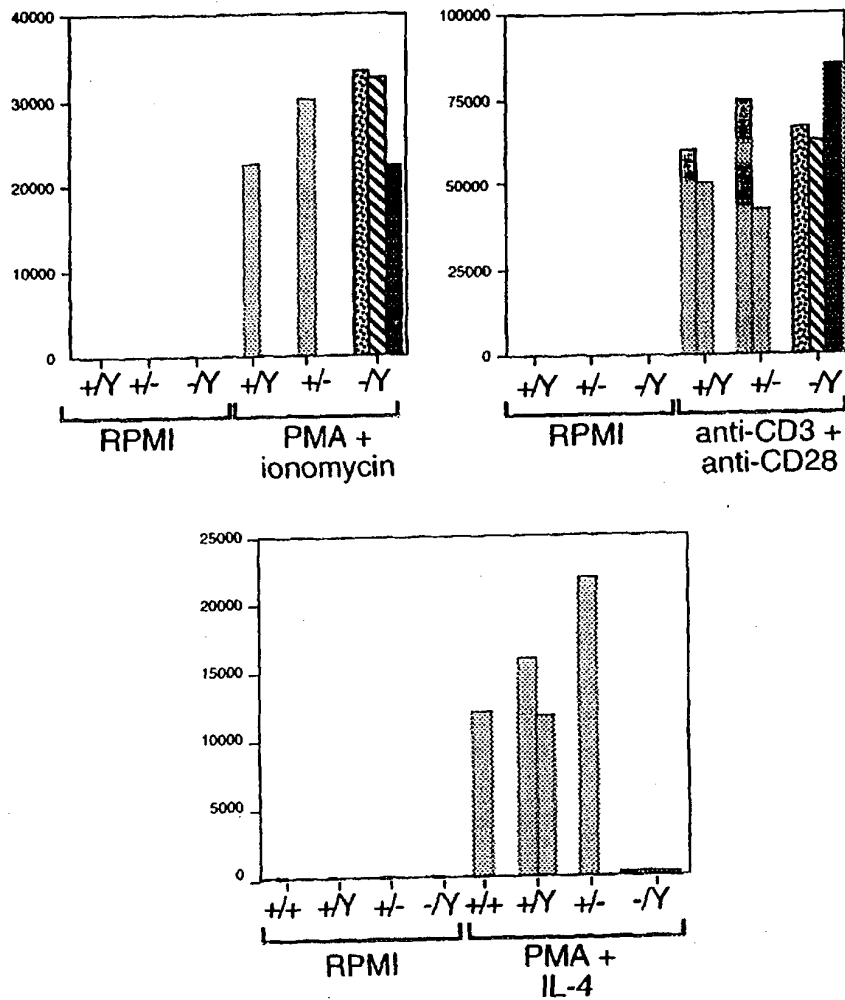


Figure 2. Thymocytes from $\gamma_C^{-/-}$ mice respond to PMA + ionomycin and to anti-CD3 + anti-CD28 (γ_C -independent stimuli), but not to PMA + IL-4 (a γ_C -dependent stimulus).

COMPARISON OF IMMUNOLOGICAL DEFECTS ASSOCIATED WITH MUTATION OF THE GENES ENCODING IL-2, IL-2R α , IL-2R β , AND γ_c

The first component of the IL-2/IL-2 receptor system to be analyzed in a murine system was IL-2 (Schorle et al. 1991). These mice develop normally during the first 3–4 weeks of life and exhibit normal thymocyte and peripheral T-cell subset composition. Some mice subsequently develop splenomegaly, lymphadenopathy, and severe anemia and die between 4 and 9 weeks of age (Sadlack et al. 1993). Of those that survive, 100% develop a form of IBD that resembles ulcerative colitis in humans (Sadlack et al. 1993). This syndrome is characterized by chronic diarrhea, intestinal bleeding, and rectal prolapse, as well as by the microscopic detection of crypt abscesses and mucosal ulcerations. Interestingly, the IL-2^{-/-} mice not only exhibit splenomegaly and lymphadenopathy, but have a high number of activated T and B cells in the colonic mucosa and drastically elevated IgG1 and autoantibody production.

Mice lacking IL-2R β expression would be expected to manifest a simultaneous loss of IL-2 and IL-15 signaling. The IL-15 receptor is analogous to the IL-2 receptor in that they both share IL-2R β and γ_c (Grabstein et al. 1994, Bamford et al. 1994, Giri et al. 1994). They differ only in the expression of different α chains, each of which has a relatively short cytoplasmic domain. So far, it appears that IL-2 and IL-15 induce identical signals when cells can respond to both cytokines (Grabstein et al. 1994, Bamford et al. 1994, Giri et al. 1994, Lin et al. 1995). Thus, if IL-2R β were restricted solely to IL-2 and IL-15 signaling, the phenotype might be expected to be quite similar to that found in IL-2 deficient mice. In IL-2R β ^{-/-} mice, thymic development was normal between 1 and 3 weeks of age, suggesting that IL-2R β is not required for T-cell development in the thymus (Suzuki et al. 1995). Interestingly, however, by 6 weeks of age, absolute numbers of thymocytes were only approximately 15% of normal levels. Since there was an increase in single positive and decrease in double positive thymocytes, this could potentially reflect general stress-induced changes. By 3 weeks of age, homozygous mice exhibited splenomegaly and lymphadenopathy, with large lymphoblastoid T cells, albeit in a normal CD4⁺CD8⁻ to CD4⁺CD8⁺ ratio. The activated CD4 cells collectively synthesized both T_H1 (γ -interferon) and T_H2 (IL-4) cytokines and stimulated synthesis of high levels of serum immunoglobulin by B cells, including autoantibodies. These included anti-erythrocyte antibodies that likely mediate the autoimmune hemolytic anemia found in IL-2R β ^{-/-} mice. Although the T cells were activated, there was no response to specific antigen nor to polyclonal activators. B- to T-cell ratios in lymph nodes were normal at three weeks of age, but by 8 weeks of age, B cells had essentially disappeared; this could be prevented by blocking the increase in CD4⁺ T cells by treatment with anti-CD4 antibodies. Thus, in the absence of IL-2R β , there is a disruption of normal lymphoid homeostasis.

Mice lacking IL-2R α expression initially have normal development of T and B

cells, but as adults, they exhibit marked lymphoid expansion with polyclonal T- and B-cell activation (Willerford et al. 1995). With time, autoimmune disorders, including inflammatory bowel disease and hemolytic anemia develop. Mice lacking IL-2R α expression are most similar to the IL-2 knockout mice, but nevertheless some differences exist (Willerford et al. 1995). Among the possible explanations for the differences would be the known ability of IL-2 to trigger certain responses via intermediate affinity IL-2 receptors (Siegel et al. 1987), which contain IL-2R β and γ_c , but not IL-2R α .

In the IL-2, IL-2R α , IL-2R β , and γ_c knockout mice, it is interesting that there is a transition point at approximately 3 weeks of age. In γ_c knockout mice, this represents the point at which the spleen enlarges and CD4⁺ T-cell expansion occurs, whereas for the IL-2R β deficient mice, this represents the point at which thymic involution occurs, and for the IL-2 knockout mice, it is at approximately 4 weeks of life that splenomegaly, lymphadenopathy, and severe anemia develop.

The fact that the phenotypes of IL-2^{-/-}, IL-2R α ^{-/-}, IL-2R β ^{-/-}, and γ_c ^{-/-} mice are all different emphasizes the complexity of cytokine-cytokine receptor systems. The principles of "cytokine pleiotropy" and "cytokine redundancy" have been reviewed in detail (Paul 1989, Leonard 1994). In addition, however, one also needs to consider the principles of "cytokine receptor subunit pleiotropy", and "cytokine receptor subunit redundancy". Cytokine pleiotropy refers to multiple actions by a single cytokine, often on multiple cell types, whereas cytokine redundancy refers to the settings where several cytokines exert overlapping actions, making it difficult to know *a priori* how severe the loss of any single cytokine will be. In effect, if a biological "function" is important, it may be subserved by more than one cytokine. Cytokine receptor pleiotropy refers to the rather well established principle that individual receptor components may act in multiple different cytokine systems. Although this has been discussed above for β_c , γ_c , and gp130, there are an ever increasing number of receptor molecules that have been recognized to be shared by multiple cytokines (see Table II). Finally, there is the interesting phenomenon of cytokine receptor redundancy, a principle perhaps best illustrated in the murine IL-3 system, wherein IL-3 signaling requires a functional IL-3R α and either the common β chain (β_c) or an IL-3 receptor specific β chain that shares 91% amino acid identity to β_c (Miyajima et al 1992). IL-3R β cannot work with IL-5 or GM-CSF and does not exist in humans. For the mouse, however, IL-3R β can fully replace β_c in mediating IL-3 action. A more confusing example appears to exist for IL-4. In this setting, it is clear that IL-4R is required for IL-4 actions, but depending on the cell type, either γ_c or an alternate additional chain (operationally denoted γ^1 and hypothesized to potentially be the IL-13R [Lin et al. 1995]) can transduce IL-4 signals. It appears that T-cell responses to IL-4 are strictly dependent on γ_c , whereas IL-4 responses on certain other cells can be mediated in the absence of γ_c and presumably require the alternate chain. It is also possible that the specific signals induced by IL-4 may depend in part on the type of receptor expressed on a given cell. If so, then

TABLE II

Cytokine receptor pleiotropy: involvement of receptor subunits as components of multiple cytokine receptors

Shared Chain	Cytokines whose receptors contain the shared chain
γ_c	IL-2, IL-4, IL-7, IL-9, IL-15
gp130	IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), IL-11, cardiotrophin-1
β_c	IL-3, IL-5, and GM-CSF
LIF receptor	LIF, OSM, and CNTF
IL-2R β	IL-2, IL-15
IL-4R	IL-4, IL-13
IL-7R	IL-7, TSLP

in this instance the principle of cytokine receptor subunit redundancy would not truly establish a redundant situation but rather would explain the basis for pleiotropic actions by IL-4. In any case, the complicated network of cytokines and cytokine receptor chains makes it difficult to predict with certainty the outcome of inactivation of any individual cytokine or receptor chain. In fact, the creation of mice defective in individual chains has allowed a greater understanding of the roles that various proteins may play.

SUMMARY

To examine the role of γ_c in lymphoid development, we have analyzed mice in which the γ_c gene was specifically inactivated by homologous recombination. These mice also serve as an animal model of human X-linked severe combined immunodeficiency (XSCID). Interestingly, γ_c knockout mice exhibited a somewhat different phenotype than humans with XSCID. Absolute T-cell numbers are greatly diminished in young $\gamma_c^{-/-}$ mice, but accumulate with age. $\gamma\delta$ T cells and NK cells are absent in $\gamma_c^{-/-}$ mice and conventional B cells are greatly diminished, yet substantial numbers of peritoneal B-1 cells are present. Since humans with XSCID have essentially no mature T cells, it is especially striking that T cells are readily apparent in $\gamma_c^{-/-}$ mice. This observation indicates that in these mice, the γ_c -dependent block in T cell development is less severe than it is in humans. It is possible but unproven that thymic stromal derived lymphopoietin, TSLP, contributes to thymocyte development in these mice. Since B-cell numbers are normal in humans with XSCID, it is also striking that $\gamma_c^{-/-}$ mice paradoxically exhibit greatly diminished numbers of B cells. This likely indicates that IL-7 signaling plays a critical role in pre-B cell maturation in mice but is less important in humans. Thus, the abnormalities observed in $\gamma_c^{-/-}$ mice have provided clues to assist in dissecting the

role of cytokines and their receptors in lymphoid development and have also identified interesting differences in the regulation of this process in mice and humans.

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Skeletal dysplasias, growth retardation, reduced postnatal survival, and impaired fertility in mice lacking the SNF2/SWI2 family member ETL1

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Abstract

The mouse *Etl1* gene encodes a nuclear protein belonging to the rapidly growing SNF2/SWI2 family. Members of this family are related to helicases and nucleic-acid-dependent ATPases and have functions in essential cellular processes such as transcriptional regulation, maintenance of chromosome stability and various aspects of DNA repair. The ETL1 protein is expressed from the two-cell stage onwards, throughout embryogenesis in a dynamic pattern with particularly high levels in the thymus, epithelia and the nervous system and in most adult tissues. As a first step to address the role of ETL1 in cells and during development, we inactivated the gene by homologous recombination. ES cells and mice lacking detectable ETL1 protein were viable, indicating that ETL1 is not essential for cell survival or for embryonic development. However, mutant mice showed retarded growth, peri/post natal lethality, reduced fertility and various defects in the sternum and vertebral column. Expressivity and penetrance of all observed phenotypes were influenced by the genetic background. Isogenic 129Sv^{Pas} mice lacking ETL1 had a severely reduced thoracic volume, which might lead to respiratory failure and could account for the high incidence of perinatal death on this genetic background. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Enhancer trap; ETL1; SNF2/SWI2 protein; Targeted mutation; Skeleton

1. Introduction

The mouse *Etl1* (enhancer-trap-locus 1) gene encodes a nuclear protein (Soininen et al., 1992; Schoor et al., 1993) belonging to the rapidly growing SNF2/SWI2 family. Members of this family are characterized by a domain of approximately 400 amino acids related to nucleic-acid-dependent ATPases and helicases (Eisen et al., 1995). Based on phylogenetic data and sequence similarities, members of the SNF2/SWI2 family can be subdivided into distinct subfamilies of proteins with proposed similar functions and activities (Eisen et al., 1995). Known functions include participation in transcriptional control, various types of DNA repair, cell cycle control and mitotic chromosome segregation (reviewed in Carlson and Laurent, 1994). It has been speculated that a common property of members of this family is to utilize ATP hydrolysis to dissociate protein-DNA complexes (Auble et al., 1994; Auble et al., 1997). The founding member of this protein family, SNF2/SWI2, is part of a large multimeric complex in yeast that

plays a key role in the regulation of transcription by remodeling chromatin structure (for reviews see: Peterson and Tamkun, 1995; Peterson, 1996). In *Drosophila*, the SNF2 homologue *Brm* (Kennison and Tamkun, 1988; Tamkun et al., 1992) is required for the transcriptional activation of multiple homeotic genes of the Antennapedia and bithorax complexes (Tamkun et al., 1992), and of other genes including the segmentation gene *engrailed* (Brizuela et al., 1994).

Mammalian *Brm* homologues include mBRG1 (Randazzo et al., 1994) and mBRM (Muchardt and Yaniv, 1993) in mouse as well as hBRG1 (Khavari et al., 1993) and hBRM in human (Muchardt and Yaniv, 1993). These proteins are components of two distinct multimeric complexes that enhance the transcriptional activation exerted by nuclear steroid hormone receptors (Khavari et al., 1993; Muchardt and Yaniv, 1993; Chiba et al., 1994; Ichinose et al., 1997), and interact with the retinoblastoma protein (Dunaief et al., 1994; Singh et al., 1995; Trouche et al., 1997). In addition, mBRG1 has recently been shown to be essential for the viability of F9 embryonic carcinoma cells (Sumi-Ichinose et al., 1997). Mice carrying mutations in the mBRM gene develop normally. However absence of mBRM leads to altered control of cellular proliferation in vitro and in vivo (Reyes et al., 1998). Other mammalian

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SNF2/SWI2 family members include CHD-1, ERCC6 and the human and mouse homologues of RAD54, respectively (Kanaar et al., 1996). CHD-1 contains, in addition to the helicase domain, a chromodomain and a DNA-binding region (Delmas et al., 1993). ERCC6 is required for the preferential repair of lesions from the transcribed DNA strand and mutations in ERCC6 cause Cockayne's syndrome which is characterized by hypersensitivity to ultraviolet light, growth retardation and neurological degenerations (Troelstra et al., 1992). Disruption of mouse RAD54 increases the susceptibility to ionizing radiation and reduces homologous recombination, consistent with a defect in double strand DNA break repair (Essers et al., 1997).

Etl1 was one of the first identified mammalian members of the SNF2 family (Korn et al., 1992; Soininen et al., 1992). The ETL1 protein is localized in the nucleus and is expressed in a complex pattern throughout embryogenesis. During preimplantation development, ETL1 expression shows a transient surge at the two-cell stage, coinciding with the activation of the embryonic genome. In blastocysts, when the first differentiation in extra-embryonic and embryonic cell lineages becomes evident, high levels of ETL1 are present in inner cell mass cells giving rise to the embryo proper, whereas trophectoderm cells contain low levels. ETL1 is expressed ubiquitously in the embryo during gastrulation, and is differentially expressed at later stages with highest levels in the developing CNS, thymus and epithelia (Soininen et al., 1992; Schoor et al., 1993). Postnatally, ETL1 is expressed at lower and more variable levels in virtually all tissues (Schoor and Gossler, unpublished data).

As a first step in analyzing the function of ETL1, we generated by homologous recombination, ES cells and mice lacking the ETL1 protein. Homozygous mutant ES cells were viable and showed no obvious defects in morphology, proliferation and sensitivity to UV and γ -radiation. Homozygous mutant mice were growth retarded and showed a decreased postnatal viability as well as impaired fertility. Abnormalities of the sternum and vertebral column were detected that represented dysplasias rather than homeotic transformations. Expressivity and penetrance of all phenotypes were influenced by the genetic background. In isogenic 129Sv^{Pas} mice lacking ETL1, the thoracic volume was greatly reduced, which might lead to respiratory failure and the high incidence of perinatal lethality observed on this genetic background.

2. Results

2.1. Targeted mutagenesis of *Etl1*

The expression of ETL1 from the earliest stage of development (Schoor et al., 1993) as well as in ES cells and in all other established mammalian cell lines analyzed so far

(Schoor and Gossler, unpublished data), suggested that ETL1 might be required for cell viability. To test this possibility in vitro, both copies of *Etl1* were consecutively mutated in ES cells by homologous recombination using two different targeting vectors (Fig. 1A,B). In both targeting vectors, a promoterless selectable marker was fused in frame to the *Etl1* coding sequence immediately downstream of the first ATG, to make use of the strong *Etl1* expression in ES cells. In the first targeting construct, most of the second exon of *Etl1* was replaced by a bifunctional *lacZ-neo* fusion gene (*leo*) (Schuster-Gossler et al., 1994) (Fig. 1A), and in the second targeting vector by the *hygromycinB phosphotransferase* (*hyg*) gene (Fig. 1B). Both constructs disrupt the *Etl1* coding sequence directly after the ATG, and thus preclude the generation of any full-length ETL1 protein. After electroporation of the *Etl1-leo* construct, seven correctly targeted clones were detected among 225 G418 resistant clones by Southern Blot hybridizations with *Etl1* probes outside the targeting vector (Fig. 1C). These ES cell clones expressed an ETL1-LEO fusion protein of the expected size (Fig. 2B) that gave rise to G418 resistance and functional β -galactosidase (data not shown). We refer to this mutant allele as *Etl1^{leo}*. The *Etl1-hyg* targeting construct was introduced into two independent heterozygous mutant clones carrying the *Etl1^{leo}* allele. In two out of 48 hygromycin resistant clones, the *Etl1^{leo}* allele was remutated and replaced by the *Etl1-hyg* construct (Fig. 1C). We refer to this allele as *Etl1^{hyg}*. After double selection with G418 and hygromycin, four out of 134 resistant clones carried both correctly targeted *Etl1* alleles (Fig. 1C). Homozygous mutant ES cell lines (*Etl1^{leo/hyg}*) were indistinguishable from heterozygous or wildtype ES cells in their morphology and proliferation rates as well as in their sensitivity to γ - or UV-irradiation (data not shown).

To test whether the introduced mutations are indeed null alleles and thus abolished expression of the ETL1 protein, *Etl1^{leo/hyg}* cells were analyzed by immunofluorescence and Western blotting with affinity purified polyclonal antibodies raised against part of the ETL1 protein carboxyterminal to the introduced deletions (Schoor et al., 1993). ETL1 was present in the nuclei of wildtype and heterozygous *Etl1^{leo}* ES cells but undetectable in *Etl1^{leo/hyg}* cells (Fig. 2A). In Western blots, anti-ETL1 antibodies detected a characteristic pattern of polypeptides (Schoor et al., 1993) in lysates of wildtype and heterozygous *Etl1^{leo}* or *Etl1^{hyg}* ES cells (Fig. 2B). All immunoreactive polypeptides were absent in lysates from *Etl1^{leo/hyg}* cells (Fig. 2B), whereas the anti- β -galactosidase antibodies detected the ETL1-LEO fusion protein in the same lysate (Fig. 2B). These results demonstrate that: (i) both mutated alleles prevent expression of detectable amounts of the ETL1 protein and thus represent true null alleles; (ii) all polypeptides detected by the polyclonal anti-ETL1 antibodies are derived from the *Etl1* gene, most likely representing posttranslationally modified and proteolytic fragments of the full length protein; and (iii)

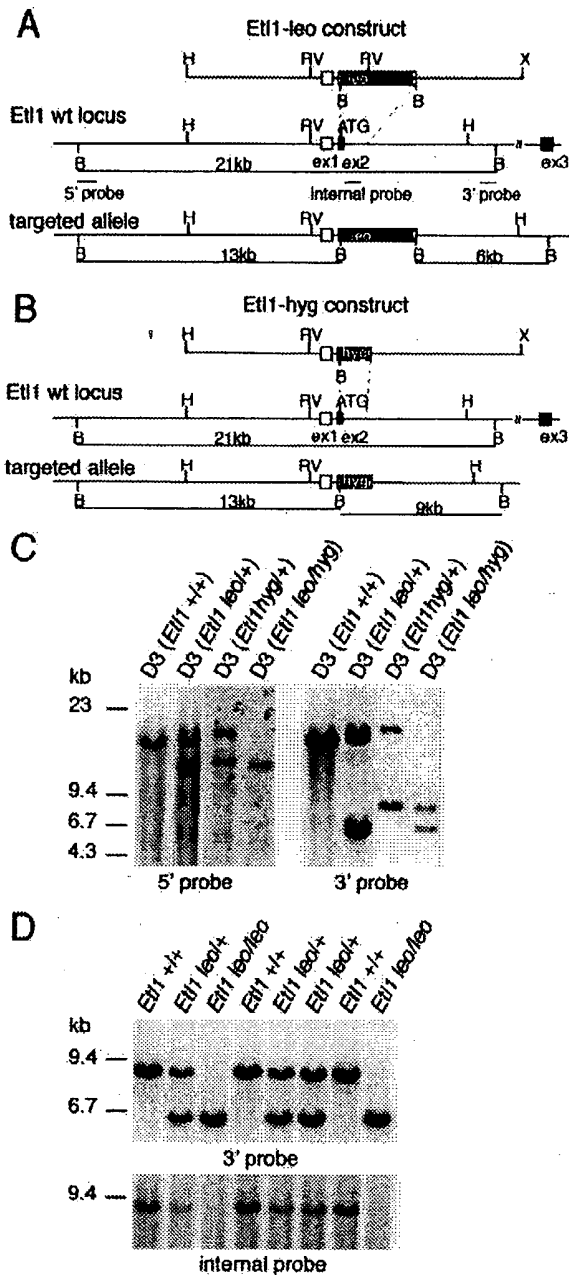


Fig. 1. Consecutive deletion of *Etl1* in ES cells and mice. (A) Structure of the *Etl1-leo* construct and of the wildtype and targeted *leo* allele. (B) Structure of the *Etl1-hyg* construct and of the wildtype and targeted *hyg* allele. The location of the probes used for Southern blot hybridizations and the expected sizes of restriction fragments of *Bam*HI digested DNA are indicated. (C) Southern blot analysis of wildtype (D3 *Etl1*^{+/+}) and *Etl1* mutant (D3 *Etl1*^{leo/+}, D3 *Etl1*^{hyg/+} and D3 *Etl1*^{leo/leo}) ES cell lines with probes from the *Etl1* locus. (D) Southern blot analysis of *Bam*HI/*Eco*RV-digested tail DNA from offspring of heterozygous *Etl1*^{leo/+} matings. The lower panel shows the same filter as the upper panel after stripping and rehybridization with an internal probe derived from the deleted regions of exon2 and intron 2. B, *Bam*HI; H, *Hind*III; RV, *Eco*RV; X, *Xba*I.

ETL1 function is not essential for ES cell viability and proliferation.

2.2. Generation and analysis of *Etl1* mutant mice

Two independent heterozygous *Etl1*^{leo} ES cell lines were introduced into the mouse germ line by blastocyst injection, and mice carrying the *Etl1*^{leo} allele were established on a mixed C57BL/6,129Sv^{Pas}, and on the isogenic 129Sv^{Pas} genetic backgrounds, respectively. Heterozygous *Etl1*^{leo/+} mice on both genetic backgrounds appeared phenotypically normal and were interbred. These matings gave rise to homozygous mutants on both genetic backgrounds (Fig. 1D). The absence of detectable amounts of ETL1 in various tissues of mutant *Etl1*^{leo/leo} mice was confirmed by immunohistochemistry and Western blot analysis of cell lysates (data not shown), demonstrating that ETL1 is not essential for apparently normal embryo development and survival. However, most homozygous mutants on both genetic backgrounds were smaller than their heterozygous and wildtype litter mates. The reduced size was apparent at late fetal stages and in newborns (not shown), and persisted through the postnatal growth period, resulting in an approximately 25% reduction in total body weight of *Etl1*^{leo/leo} mice at 5 weeks of age (Fig. 3).

Whereas *Etl1*^{leo/leo} homozygotes were present at a normal Mendelian ratio on gestational day 18 and day 1 after birth, significantly fewer homozygous mutants than expected were found after weaning on both genetic backgrounds (Table 1). To analyze when ETL1-deficient mice die, homozygous mutants on the mixed genetic background were intercrossed, and the survival of the offspring was monitored after birth. All offspring from these matings were homozygous mutants, eliminating potential competition effects that might occur in litters from heterozygous matings containing also heterozygous and wildtype siblings. From 87 born homozygous mutants, 13 died during the 1st day post partum (pp), 19 more during the 1st week, and additional 13 during the subsequent 2 weeks, resulting in a total loss of 52% of homozygous mutants in the first 5 weeks. In contrast, none of the 34 wildtype newborns on a similar mixed genetic background (C57BL/6x129Sv^{Olaf})F1 died in the first 5 weeks after birth. On the isogenic background, many homozygotes were stillborn or died within the first 12 h after birth (Table 1, and data not shown). These offspring were obtained from heterozygous matings because isogenic homozygous mutants had a very low fecundity (see below). Autopsy of homozygous mutants revealed, in a subset of these mice, various abnormalities such as hypertrophy of the heart, gastro-intestinal tumors or a rudimentary appendix (data not shown). However, no consistent cause for the postnatal mortality could be identified. To test whether immunological defects caused by aberrant lymphocyte development contribute to the observed mortality, we analyzed lymphoid organs and the composition of B and T-cells. The size and cellularity of thymi, lymph nodes

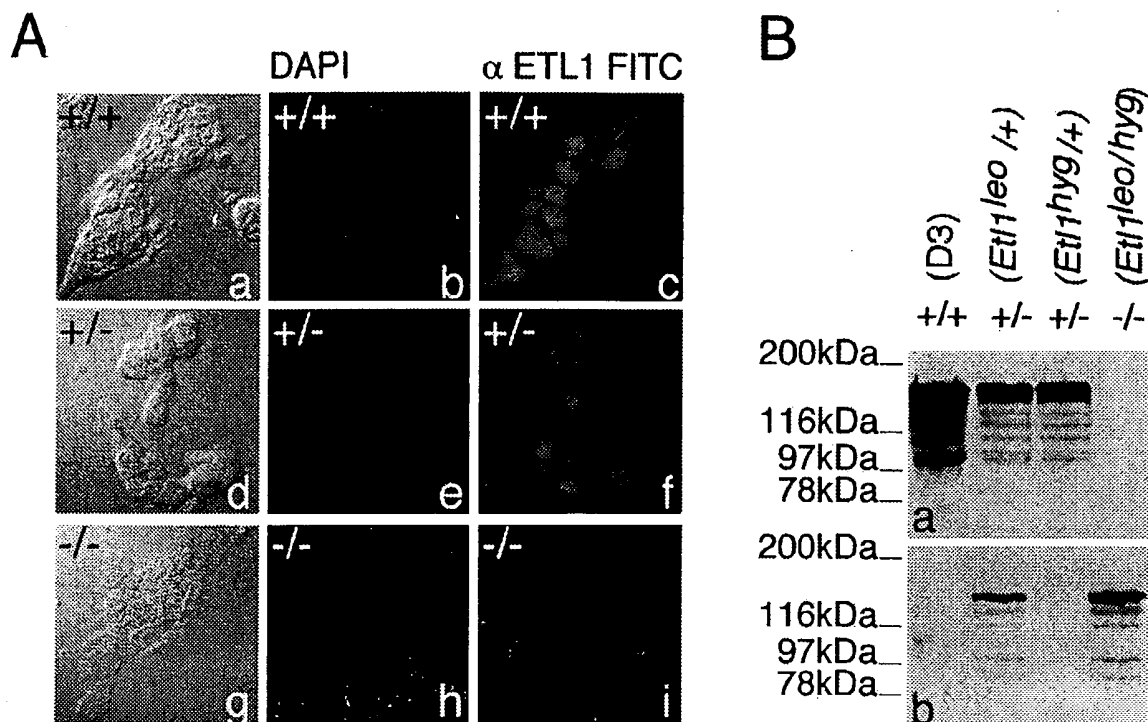


Fig. 2. *Etl1*^{leo} and *Etl1*^{hyk} are null alleles of *Etl1*. (A) Microphotographs of *Etl1*^{+/+} (a–c), *Etl1*^{leo/+} (d–f) and *Etl1*^{leo/hyg} (g–i) D3 ES cells. Differential interference contrast images (a,d,g) and DAPI (b,e,h) and FITC (c,f,i) epifluorescence images of the same cells labeled with DAPI and stained for ETL1 by indirect immunofluorescence. Note the speckled expression of ETL1 in the nucleus and the absence of ETL1 staining in *Etl1*^{leo/hyg} ES cells. (B) Western blot analysis with lysates from *Etl1*^{+/+}, *Etl1*^{leo/+}, *Etl1*^{hyg/+} and *Etl1*^{leo/hyg} ES-cell lines with polyclonal α-ETL1 (upper panel) and anti-β-Gal antibodies (lower panel). Note the absence of detectable ETL1 in *Etl1*^{leo/hyg} ES cell lysates whereas the ETL1-LEO fusion protein is detected in the same lysate.

and spleens of homozygous mutant, heterozygous, and wild-type mice were indistinguishable. Similarly, multiparameter flow fluorimetric analysis of thymocytes and lymph node cells using lymphocyte lineage specific T-cell surface markers (CD3e, CD4 and CD8) and B lymphocyte markers (Ig and CD45R) failed to reveal any qualitative or quantitative differences between mutant and wildtype mice (not shown).

On the mixed genetic background, intercrosses of homozygous mutants gave rise to offspring in the majority of cases. However, the number of progeny was reduced (aver-

age litter size 4.2 in 15 litters) compared to intercrosses of heterozygous *Etl1* mice (average litter size seven in 15 litters; $P < 0.001$). To address whether the reduced fertility could be attributed specifically to males or females, homozygous mutants of either sex were mated to wildtype mice over a period of several months. Eight out of eight homozygous mutant males and ten out of 13 homozygous mutant females were fertile and gave rise to progeny. The average number of offspring per litter derived from matings between homozygous mutant males with wildtype females was similar to control matings between heterozygous mutants. In

Table 1

Genotypes of offspring obtained from matings between heterozygous *Etl1* mutants on the mixed and isogenic background. Chi square values 15 and 24, respectively; 2 d.f.; $P < 0.001$

Stage	Number of mice genotyped	<i>Etl1</i> ^{+/+} (%)	<i>Etl1</i> ^{leo/+} (%)	<i>Etl1</i> ^{leo/leo} (%)
C57BL/6,129Sv ^{Pas} mixed genetic background				
D18 p.c.	45	10 (22)	26 (58)	9 (20)
Newborns	70	17 (24)	36 (51)	17 (24)
Adults	122	35 (29)	71 (59)	16 (13)
129Sv ^{Pas} isogenic genetic background				
Newborns	26	7 (27)	12 (48)	7 (27) ^a
Adults	119	36 (30)	70 (59)	13 (11)

^a Includes six stillborns.

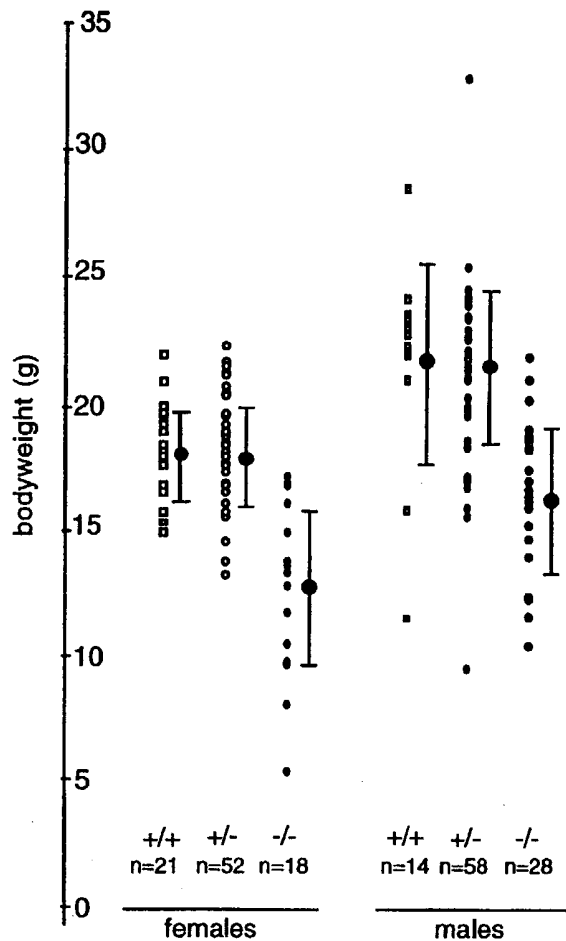


Fig. 3. Reduced body weight of *Etl1*^{leol/leol} adult mice. The weight of individual wildtype, *Etl1* heterozygous and *Etl1* homozygous adult females and males (D33–D39 pp) on the mixed background, and the median weight per group (large black dot to the right of each group) with standard deviation are shown.

contrast, the average litter size obtained from homozygous females mated with wildtype males was reduced (average litter size 3.9 in nine litters; $P < 0.01$; Table 2). On the isogenic 129Sv^{Pas} genetic background, matings between homozygous mutants resulted only in four litters with one or two pups, and all offspring were stillborn or died within the 1st day after birth. Test matings of homozygous mutants with wildtype or heterozygous mice showed that only 60% (eight out of 13) of the males and 26% (four out of 15) females gave rise to progeny. To identify a potential cause for the reproductive defect(s), we analyzed the reproductive tract and gonads of both fertile and infertile mutants of both sexes on both genetic backgrounds. In some fertile as well as infertile females, the number of oocytes was reduced (data not shown). However, no gross morphological abnormalities or histopathological changes that could account for reduced litter size or infertility of individual males or females were detected.

Table 2
Litter sizes obtained from matings with *Etl1* mutants

Litter variable	Combination of genotypes			
	m × f +/+ × +/-	m × f -/- × +/+	m × f +/+ × -/-	m × f -/- × -/-
Number litters	15	8	9	15
Average litter size	7	7.6	3.9 ^a	4.2 ^b

^a Significant difference to the average litter size in control matings; $P < 0.01$.

^b Significant difference to the average litter size in control matings; $P < 0.001$.

2.3. Skeletal dysmorphologies in *Etl1* mutant mice

To address whether the loss of ETL1 function might lead to patterning defects similar to the *brm* mutation in *Drosophila*, we analyzed skeletal preparations from ETL1-deficient mice. On the mixed background, various skeletal abnormalities were found in a subset of the heterozygous and homozygous mutants, but not in the wildtype controls (Table 3), suggesting that the reduction or loss of ETL1 causes skeletal dysplasias with incomplete penetrance and variable expressivity. In wildtype newborns, seven pairs of ribs are fused bilaterally symmetrically to the sternum dividing it into the manubrium, four clearly detectable sternbrae and xiphoid process. In three of the 35 analyzed *Etl1*^{leol/+} mice, the eighth rib was also fused unilaterally to the sternum (Fig. 4A), while the overall number of ribs was unaltered. This phenotype was not found in homozygous mutant animals. However, in three out of the 17 analyzed *Etl1*^{leol/leol} mice, the thoracic ribs were fused asymmetrically in an alternating manner to the sternum (Fig. 4B,C). In addition, the sternbrae were incompletely ossified in these mice (arrowheads in Fig. 4B,C), and the most severely affected animal also exhibited fusions between the tenth and 11th rib, and the fourth and fifth lumbar vertebra, respectively (data not shown).

All observed phenotypes show incomplete penetrance on the mixed background. To evaluate effects of the genetic

Table 3
Skeletal dysplasias in *Etl1* mutant mice on the mixed background

Condition	<i>Etl1</i> genotype		
	+ / +	leol / +	leol / leol
Number of analyzed mice	11	35	17
Mice with			
8/7 vertebro-sternal ribs	0	3	0
Asymmetrical fusion of ribs to sternum	0	0	3
Fused ribs	0	0	1
Floating ribs	1	0	1
Processus spinosus on T2 missing	0	3	0
Fused lumbar vertebra	0	0	1

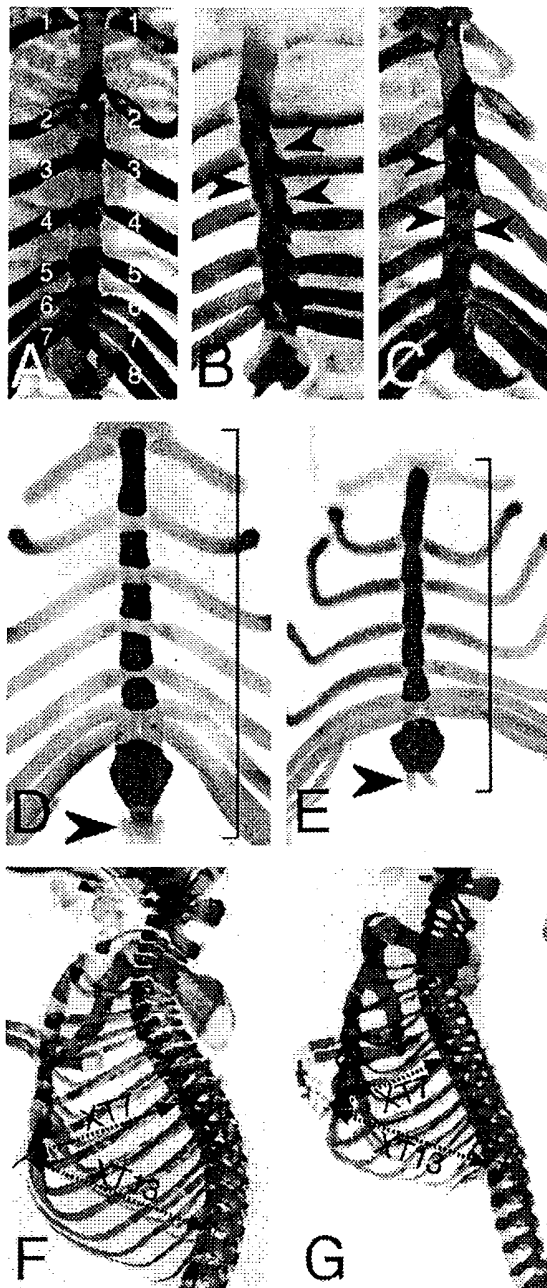


Fig. 4. Skeletal dysplasias in *Etl1* mutant newborns. Unilateral fusion of the eighth rib to the sternum in a *Etl1*^{leol/+} mouse (A), and asymmetrical fusions of the ribs to the sternum in homozygous *Etl1* mutant mice (B,C) on the mixed 129Sv/C57BL/6 genetic background. Frontal views of dissected sternums of 129Sv wildtype (D) and isogenic *Etl1*^{leol/leol} (E) mice. Note the strongly reduced length of the sternum and the split xiphoid process (arrowhead) in (E). Lateral view of the rib cage of a 129Sv wildtype (F) and an isogenic *Etl1*^{leol/leol} littermate (G). Note the absence of the curvature of the vertebral column and the reduced size of the ribcage in (G), as measured by the distance from the xiphoid process to the seventh (XT7) and 13th (XT13) thoracic vertebra.

background on the skeletal phenotypes, skeletal preparations from 14 wildtype, 16 *Etl1*^{leol/+} and ten *Etl1*^{leol/leol}

newborns or stillborns on the 129Sv background were analyzed. On this genetic background, skeletal defects of the sternum and vertebral column were different from the mixed background. All 10 *Etl1*^{leol/leol} mice had a drastically shortened sternum (Fig. 4E,G) and the xiphoid process was split or bifid (arrowhead in Fig. 4E), whereas it was fused in all analyzed wildtype animals and in 14/15 heterozygous animals (arrowhead in Fig. 4D). The ribs were shortened and connected to the sternum rectangularly (Fig. 4E) in contrast to the normally acute-angled connections (Fig. 4D). In addition, the curvature of the vertebral column was obviously altered in half of the homozygous mutants. The thoracic kyphosis was absent in five newborns and two animals also lacked the cervical lordosis (compare Fig. 4F,G). The missing curvature of the vertebral column together with the shortening of the sternum and ribs resulted in a strongly reduced size of the thorax, as measured by the distance between the xiphoid process and the seventh (XT7) or 13th thoracic vertebra (XT13), respectively (Fig. 4F,G; Table 4). The different skeletal alterations that were observed in mutants on the mixed genetic background and on the isogenic 129Sv ETL1 background, suggest that the *Etl1* mutation is strongly modified by the genetic background.

3. Discussion

ETL1 was identified as one of the first mammalian members of the SNF2/SWI2 family of proteins (Korn et al., 1992; Soininen et al., 1992; Schoor et al., 1993). These proteins share a domain with similarities to helicases and nucleic-acid-dependent ATPase, and are involved in various basic cellular functions such as DNA repair, cell-cycle control and transcriptional regulation (reviewed in Carlson and Laurent, 1994). Based on molecular phylogenetic analysis, the SNF2/SWI2 family has been subdivided into distinct subfamilies, and it has been proposed that functions are conserved within, but not among, subfamilies (Eisen et al., 1995). ETL1 forms a distinct subgroup together with the yeast protein FUN30 (Clark et al., 1992). Besides the helicase-related SNF2 domain, ETL1

Table 4

Anterior-posterior and dorso-ventral thoracic dimensions in isogenic *Etl1*^{+/+}, *Etl1*^{leol/+} and *Etl1*^{leol/leol} newborn mice^a

Thoracic dimension	<i>Etl1</i> genotype		
	+/+	leol/+	leol/leol
Number of analyzed skeletons	3	8	5
Distance between			
Clavicle and xiphoid process	55.0 (0)	53.2 (2.2)	47.0 (3.5)
Xiphoid process and T13	74.0 (1.7)	71.6 (6.1)	64.2 (3.2)
Xiphoid process and T7	54.7 (2.5)	56.6 (2.3)	47.6 (5.3)

^a The distances between indicated skeletal elements are given in arbitrary units as average sizes and standard deviations are shown in parentheses.

contains additional short stretches of significant homology (26–38% identity, 40–61% similarity) to the SNF2 subfamily of CHD proteins in fly (Stokes and Perry, 1995), chick (Griffiths and Korn, 1997) mouse (Delmas et al., 1993) and man (Woodage et al., 1997). However, this homology is outside of the chromo- and DNA-binding domains and no specific functions have as yet been assigned to these conserved regions. Thus, although the amino acid sequence clearly identifies ETL1 as a SNF2 family member, it does not provide clues as to its specific biological function. The early and widespread expression of ETL1 in the developing embryo (Soininen et al., 1992; Schoor et al., 1993), as well as in ES cells and all other established cell lines ($n = 12$) analyzed thus far (Schoor and Gossler, unpublished data), is consistent with potential roles for ETL1 in basic cellular functions potentially affecting development. However, the expression does not point to a particular developmental stage or process that might require this function.

As a first step to identify and analyze potential function(s) of ETL1 in cells and during development, the *Etl1* gene was inactivated by homologous recombination. Our analysis of homozygous mutant ES cells demonstrates that ETL1 function is dispensable for cell proliferation and viability. These results indicate that ETL1 is not an essential component of the basal transcription machinery, or critical for cell-cycle progression in ES cells. This is in contrast to the related SNF2 β -BRG1, which is required for the survival of F9 EC cells (Sumi-Ichinose et al., 1997), and to SNF α -mBRM, which is required for cell cycle arrest in the G₀/G₁ phase in response to cell confluency or UV radiation (Reyes et al., 1998). Some SNF2 family members have been implicated in DNA repair, and ES cells lacking mHR54 show reduced resistance to ionizing radiation (Essers et al., 1997). However, homozygous mutant *Etl1* ES cells showed no differences in their survival rates after treatment with γ - or UV-radiation as compared to heterozygous and wildtype controls (data not shown), suggesting that ETL1 is not essential for double strand break repair or excision repair of pyrimidine dimers. However, this does not rule out a requirement for ETL1 in cellular responses to other DNA damaging agents. This result also suggests that members of the proposed SNF2/SWI2 subfamilies (Eisen et al., 1995) do not necessarily share identical function(s), because the only other known member of the ETL1 subfamily, the yeast protein FUN30, has been implicated in mediating cellular responses to UV radiation (Barton and Kaback, 1994).

The loss of ETL1 in mice caused a specific set of defects, that occurred with variable expressivity and incomplete penetrance, depending on the genetic background. These variations are unlikely due to residual ETL1 activity because: (i) the protein was disrupted immediately after the first ATG; and (ii) no ETL1 protein was detected in homozygous mutant cells, embryos or adult tissues. From heterozygous intercrosses the expected number of homozygous mutant embryos were found shortly before and after

birth. However, on both genetic backgrounds analyzed, the lack of ETL1 affected body weight and viability, as well as fertility, but no consistent pathological alterations could be associated with these phenotypes.

In *Drosophila*, the maintenance of homeotic (HOM-C) gene expression is controlled by two antagonistic groups of genes, the Polycomb group (Pc-G) and the trithorax group (Trx-G) (Paro, 1990; Kennison and Tamkun, 1992; Kennison, 1993). Mutations in Pc-G and Trx-G genes lead to deregulated maintenance of homeotic gene expression and thus to homeotic transformations (Paro, 1990; Tamkun et al., 1992; Brizuela et al., 1994). This regulatory interaction is evolutionary well-conserved, since mutations in mouse homologues of Pc-G and Trx-G genes also lead to deregulated Hox gene expression resulting in homeotic transformations of the axial skeleton (van der Lugt et al., 1994; Yu et al., 1995; Akasaka et al., 1996; van der Lugt et al., 1996; Core et al., 1997). Since *Etl1* has sequence similarity with *brm* (Soininen et al., 1992), a *Drosophila* Trx-G gene implicated in maintenance of HOM-C expression (Tamkun et al., 1992; Brizuela et al., 1994; Elfiring et al., 1998), it appeared possible that ETL1 could also be required for Hox gene regulation, and that the loss of ETL1 function might cause defects of the vertebral column and ribs consistent with such a role. Although the fusion of the eighth rib to the sternum in heterozygous mutants could be regarded as homeotic transformation, the normal position of other axial markers is not consistent with this view. In addition, the absence of homeotic transformations in homozygous *Etl1* mutants does not support a function for ETL1 in maintaining Hox gene expression early during development, when the identity of vertebrae is determined (Kieny et al., 1972; Chevallier et al., 1977). In *Drosophila*, *brm* was isolated as a suppressor for homeotic transformations caused by *polycomb* mutations (Kennison and Tamkun, 1988; Tamkun et al., 1992). The inactivation of *Etl1* will now allow for testing whether the loss of ETL1 function can modulate the phenotypes of mouse polycomb homologues *bmi1*, *mell18* and *M33*, that also lead to posterior homeotic transformations (van der Lugt et al., 1994; Akasaka et al., 1996; Core et al., 1997).

The sternum phenotype of *Etl1* mutant mice demonstrates that *Etl1* function is required later for normal skeletal development. Whereas the ribs and vertebra derive from the paraxial mesoderm, the sternum develops late in organogenesis from derivatives of the lateral mesoderm. At embryonic day 12, the lateral mesoderm gives rise to a pair of condensations between the clavicle and the first rib pair. During the next 2 days, these condensations extend caudally and form the sternal bands on each side of the heart. These bands gradually move together and differentiate into hyaline cartilage. The growth of ribs and sternal band are initially independent from each other. The first pair of ribs fuses with the sternal bands on embryonic day 13. On day 14, seven pairs of ribs are in contact with the sternal bands which begin to fuse at their anterior end. By day 16, the sternal bands have

fused along their entire anterior-posterior extent, and the posterior ends are joined and project beyond the attachment of the seventh rib as a well defined single xiphoid process. The ossification of the intercostal regions of the sternum then gives rise to the sternbrae (Green and Green, 1942; Chen, 1952). The asymmetrical alignment of the ribs to the sternum on the mixed background suggests that growth and/or fusion of the sternal bands does not occur in a coordinated fashion in homozygous *Etl1* mutants. This misalignment probably leads to the reduction of the ossification of the sternbrae, since the rib tips prevent ossification of the sternum (Chen, 1953). The size reduction and split xiphoid process in isogenic homozygous mutants also suggest that growth and fusion of the sternal bands are impaired. Sternum defects similar to those in *Etl1* mutants have been described for mice with mutated, or misexpressed Hox genes (e.g. Kaur et al., 1992; Pollock et al., 1992; Condie and Capecchi, 1993; Jeannotte et al., 1993; Ramirez-Solis et al., 1993; Horan et al., 1994; Suemori et al., 1995; Barrow and Capecchi, 1996), in *En1* mutants (Wurst et al., 1994), and in mice lacking BMP5 (Green and Green, 1942; Kingsley, 1994). Whether ETL1 interacts with any of these genes, and the molecular mechanism(s) by which ETL1 acts in sternum development, remains to be resolved. Approximately half of the *Etl1* mutant newborns on the 129Sv background also lacked the normal curvature of the vertebral column, in particular the thoracic kyphosis. Together with the shortened sternum, this lead to a severely reduced thoracic size, which is likely to diminish the respiratory volume. Thus, respiratory distress might account for the high incidence of perinatal lethality seen on the 129Sv genetic background. This aspect of the *Etl1* phenotype resembles the autosomal recessive Jeune syndrome in humans (asphyxiating thoracic dystrophy, ATD; OMIM accession number 208500) (Jeune et al., 1955), which results in fatal neonatal outcome in most affected individuals.

The absence of an absolute requirement for ETL1 in ES cells and during development and the low penetrance and expressivity of the phenotype could indicate that other SNF2 family members can compensate in part for the loss of ETL1 function. At least four other SNF2 family members that show strong sequence homology to ETL1 have been isolated in the mouse: CHD1 (Delmas et al., 1993), mHR54 (Kanaar et al., 1996), mBRM (Muchardt and Yaniv, 1993) and mBRG1 (Randazzo et al., 1994). The inactivation of mHR54 and mBRM have shown that both proteins are dispensable for embryonic development (Essers et al., 1997; Reyes et al., 1998) and no overlap was found with the phenotype of mice lacking ETL1 that could be indicative of potential common function(s). A subset of adult mBRM mutant mice show a moderate increase in body weight, most likely due to increased cell proliferation (Reyes et al., 1998). This rather opposite phenotype, compared to the decreased body weight in *Etl1* mutant mice, might argue against overlapping functions of ETL1 and mBRM. The absence of an apparent embryonic phenotype in mBRM deficient mice has

been speculated to be the result of a functional rescue by mBRG1 (Reyes et al., 1998). Since the expression of mBRG1 throughout development in many cell types and tissues shows considerable overlap with *Etl1* (Randazzo et al., 1994; LeGouy et al., 1998), and mBRG1 contains all conserved protein motifs present in ETL1, it is conceivable that mBRG1 might also compensate for the loss of ETL1. However, the analysis whether the unexpected mild embryonic phenotypes observed after inactivation of mammalian SNF2 homologues can be accounted for by the compensation of other family members as mBRG1, has to await their inactivation in mice and the subsequent generation of compound mutants.

4. Material and methods

4.1. Construction of the vectors for homologous recombination

Two different vectors were constructed for consecutive homologous recombination at the *Etl1* locus. To mark the mutated allele a *lacZ-neo* fusion (*leo*) that gives rise to a bifunctional β -Gal-neomycin-phosphotransferase fusion protein (Schuster-Gossler et al., 1994) was used. The vector *Etl1-leo* was constructed for the targeting of the first allele. The selection/reporter cassette was cloned in the appropriate ORF immediately behind the putative translation initiation site. Briefly, a λ clone containing the first two exons of *Etl1* was isolated from a genomic 129Sv library. A 474bp *Asp700/HindIII* fragment containing the endogenous ATG and a 819 bp *XmaI* fill in/*Clal* fragment from the vector pLRacZpA/MCIneoA (Rüpping, 1994) containing the 5' end of the *lacZ* gene were ligated in the gene targeting vector pTFu (Schuster-Gossler et al., 1994), in which the *cfos* splice acceptor and the 5' end of the *lacZ* gene was removed by *HindIII/Clal* digestion. This results in a fusion of the *leo* gene 4 nucleotides behind the endogenous ATG, maintaining the ORF. The transition between *Etl1* and *lacZ* was verified by sequencing analysis. A 129Sv genomic 6 kb *HindIII* fill in fragment was fused in a *XbaI* fill in site in the polylinker downstream of the *leo* gene as 3' arm and a 7 kb *HindIII* fragment was fused at the *HindIII* site as 5' arm of the construct (Fig. 1A).

The vector *Etl1-hyg* was used for the consecutive mutation of the second *Etl1* allele. The vector pHAhygP was constructed from the vectors pHA56 and pHA58 (te Riele et al., 1990). This vector contains the promoterless hygromycin phosphotransferase (*hygB*) gene followed by the PGK poly A sequence. From this vector, the *hygB* pA cassette was excised by *SacI* and *HindIII* digests and the ends were filled in by T4 ligase. The *hygB* pA cassette was ligated in the vector *Etl1-leo* in which the *leo* cassette was removed by *BamHI* digestion and subsequent fill-in reaction. This vector contains the *hygBpA* gene in the appropriate ORF 13 nucleotides behind the putative ATG (Fig. 1B).

4.2. ES cell culture

D3 ES cells were maintained as described previously (Gossler et al., 1989). The different constructs were introduced in D3 ES cells by electroporation. 1×10^7 ES cells were electroporated with 10 μ g of each construct in a volume of 0.8 ml PBS using a Bio-Rad gene pulser set at 500 μ F/240 V. Cells were immediately plated on ten gelatinized 100 mm dishes and were cultured in BRL conditioned LIF media (30% BRL, 15% FCS, 1/10000 LIF). Thirty-six to 48 h after electroporation, the transfected ES cells were selected with 300 μ g/ml G418 and/or 150 μ g/ml Hygromycin B on gelatinized plates without feeder cells in BRL/LIF media for 8–10 days. Antibiotic resistant cells were picked and were immediately split on 96 well master plates with feeder layer and gelatinized replica plates. On both plates, the ES cells were further expanded in ES media without selection. The master plates were frozen in ES cell media containing 20% FCS, 10% DMSO when sub confluent, whereas the replica plates were expanded to confluence and used for DNA preparation and restriction digest analysis essentially as described in Ramirez-Solis et al. (1993).

4.3. Cell proliferation and survival assays

For comparing cell proliferation, 5×10^6 *Etl1^{leol+}* and *Etl1^{leolhyg}* ES cells, respectively, were plated in triplicates on 35 mm dishes containing mitomycin C-treated embryonic fibroblasts using ES cell medium supplemented with recombinant LIF. The medium was changed on the next day, and cells were washed, trypsinized and counted on the subsequent day. From these cell suspensions, 5×10^6 ES were plated on fresh 35 mm dishes as above, and counted on the 2nd day after plating cell counts were obtained from four consecutive rounds of plating.

For the analysis of cell survival after UV and γ -irradiation, 5×10^6 *Etl1^{leol+}* and *Etl1^{leolhyg}* ES cells, respectively, were plated in triplicates as single cell suspensions on gelatin-coated 35 mm dishes in six well plates in ES cell media supplemented with recombinant LIF. On the next day, cells were washed once with PBS and plates were UV-irradiated without PBS and lid using a Stratalinker (Stratagene) in the range of 0–100 J/m² and γ -irradiated using a Cesium source in the range of 0–400 R/min. Medium was added back to the wells and the surviving colonies were counted after 8 days of culture.

4.4. Immunological methods

Western Blot analysis, immunofluorescence and immunohistochemistry on sections and whole embryos with the α -ETL1 and α - β Gal antibodies were done as described previously (Schoor et al., 1993).

4.5. Generation of transgenic mice

Chimeras were generated by injection of two independent

targeted *leo* ES cell lines into C57BL/6 blastocysts that were subsequently transferred to (C57BL/6xBALBc)F1 pseudo-pregnant females. Germline chimeras were obtained and crossed to either C57BL/6 or to 129Sv females to establish the mutation on a mixed or isogenic background, respectively.

4.6. Skeletal preparation

Skeletons were prepared essentially as described by (Kessel and Gruss, 1991). The skin and visceral organs were removed and the animals were fixed for several days, first in 100% ethanol and then in acetone at room temperature. Afterwards, the specimens were stained for 2–14 days in 0.15% alcian blue, 0.15% alizarin red, 5% acetic acid in ethanol at 37°C and cleared in Glycerin/KOH solution. The skeletons were stored and photographed in glycerol.

4.7. Analysis of lymphopoietic cells

T and B cell surface differentiation markers were evaluated by multiparameter flow cytometry using conventional procedures and the following directly labeled antibodies as described (Christianson et al., 1997): 145-2C11 anti-CD3 ϵ -FITC; 56-6.72 anti-CD8a-PE; GK1.5 anti-CD4-CY3; 53-6.7 anti-CD8a-PE; clone RA3-6B2 anti-CD45R/B220-PE, clone H1.2F3 anti-CD69 biotin/SA, and goat anti-mouse immunoglobulin (Ig), obtained from Pharmigen Inc. (San Diego CA) or prepared by The Jackson Laboratory Flow Cytometry Service. Viable cells were gated by propidium iodide exclusion, and analysis was performed using LYSIS II software on a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA).

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Gtl2^{lacZ}, an insertional mutation on mouse Chromosome 12 with parental origin-dependent phenotype

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Abstract. We have produced a transgenic mouse line, *Gtl2^{lacZ}* (Gene trap locus 2), that carries an insertional mutation with a dominant modified pattern of inheritance: heterozygous *Gtl2^{lacZ}* mice that inherited the transgene from the father show a proportionate dwarfism phenotype, whereas the penetrance and expressivity of the phenotype is strongly reduced in *Gtl2^{lacZ}* mice that inherited the transgene from the mother. On a mixed genetic background this pattern of inheritance was reversible upon transmission of the transgene through the germ line of the opposite sex. On a predominantly 129/Sv genetic background, however, transgene passage through the female germ line modified the transgene effect, such that the penetrance of the mutation was drastically reduced and the phenotype was no longer obvious after subsequent male germ line transmission. Expression of the transgene, however, was neither affected by genetic background nor by parental legacy. *Gtl2^{lacZ}* maps to mouse Chromosome 12 in a region that displays imprinting effects associated with maternal and paternal disomy. Our results suggest that the transgene insertion in *Gtl2^{lacZ}* mice affects an endogenous gene(s) required for fetal and postnatal growth and that this gene(s) is predominantly paternally expressed.

Introduction

The maternal and paternal genomes of mammalian embryos are functionally not equivalent, a phenomenon referred to as “genomic imprinting” (Solter 1988; Reik 1992; Surani 1993; Surani et al. 1993; Efstratiadis 1994). Diploid mouse embryos that carry either two paternal or two maternal genomes fail to complete embryogenesis and die during early postimplantation development with opposite and complementary phenotypes (McGrath and Solter 1984; Surani et al. 1984, 1986, 1990). Genetic studies have defined most of the chromosomal regions in the mouse genome that are associated with imprinting (Cattanach and Kirk 1985; Cattanach and Beechey 1990; Cattanach and Rasberry 1994). It is thought that these regions contain genes that are expressed during embryogenesis almost exclusively from the maternal or paternal allele and that this differential gene expression is brought about by a reversible epigenetic modification imposed during gametogenesis. Several mouse genes that are differentially expressed from the paternal or maternal allele and are required for normal embryonic growth and viability have been isolated and were mapped to previously identified imprinted regions (Barlow et al. 1991; Bartolomei et al. 1991; DeChiara et al. 1991; Leff et al. 1992).

Similar to endogenous genes, transgenes can be subject to parental origin-dependent effects that lead to differences in the expression and/or methylation state (Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987; Allen et al. 1990; Chaillet et al. 1991).

Whereas sequences in the transgene per se can be responsible for the imprinting effect (Chaillet et al. 1995), in most cases these effects are thought to be due to the chromosomal position of the insertion site, which exerts its influence on the transgene. This idea is further supported by the parental origin-dependent effects observed in the transgene insertional mutation acrodysplasia (*Adp*), in which parental legacy controls expression of the transgene as well as expression of phenotypic abnormalities caused by the transgene insertion (DeLoia and Solter 1990).

Here we report on a transgene insertional mutation, Gene-trap-locus-2-lacZ (*Gtl2^{lacZ}*), that affects fetal and postnatal growth. The phenotype is inherited in a modified dominant manner, whereas the expression of the transgene is not affected by parental legacy. The transgene insertion maps to mouse Chromosome (Chr) 12 in a region recently demonstrated to show imprinting effects. Our results support these findings and are consistent with the idea that the transgene insertion in *Gtl2^{lacZ}* mice affects a gene on mouse Chr 12 predominantly expressed from the paternal allele.

Materials and methods

Establishment of transgenic mouse line *Gtl2^{lacZ}*. The 129/Sv-derived ES cell line D3/gt 216, which carries the gene trap vector pGTi integrated in its genome (Schuster-Gossler et al. 1994), was injected into C57BL/6 blastocysts as described (Gossler et al. 1986). Male chimeras were mated to C57BL/6 females, and heterozygous transgenic mice were identified by Southern blot hybridizations (see below) of tail DNAs from agouti offspring (that is, those derived from the ES cell line). Heterozygous transgenic males obtained from these matings were bred to 129/Sv or to (C57BL/6 × BALB/c)F₁ females.

Cloning of the insertion site and typing of transgenic animals. A genomic library in λEMBL3 was made by standard techniques (Sambrook et al. 1989) from partially *Nde*II-digested genomic DNA obtained from cell line D3/gt 216 and was screened with pGTi (Schuster-Gossler et al. 1994) as a probe. Positive clones were plaque purified and inserts subcloned into pBSII SK (Stratagene). A unique 1.8-kb genomic *Pst*I fragment detected a restriction fragment length polymorphism in *Eco*RI-digested genomic mouse DNA with a 10-kb band corresponding to the wild-type allele and a 9-kb band to the transgenic allele.

Mice were typed by Southern blot hybridizations of *Eco*RI-digested tail DNA with a 3-kb *lacZ* probe or the 1.8-kb *Pst*I fragment. Heterozygous embryos were identified by Southern blot hybridizations or staining for β-galactosidase activity as described (Gossler and Zachgo 1993).

Interspecific backcross analysis. 100 DNA samples prepared from the spleen of an interspecific backcross progeny involving the laboratory strains C57BL/6 and STF (a moderately inbred strain derived from the *Mus spretus* species) were used for the chromosomal mapping of the transgenic insertion *Gtl2^{lacZ}*. These samples were taken from the EUCIB resource (Breen et al. 1994), and were typed for *Gtl2^{lacZ}* genotype with a 1-kb probe

approximately 10 kb upstream the insertion site. Linkage and genetic distances were computed with the MBx software (Breen et al. 1994).

Results

Transgenic mouse line *Gtl2^{lacZ}* was derived from germ line chimeric males obtained by blastocyst injection of the gene trap ES cell line gt216 (Schuster-Gossler et al. 1994). Approximately half of the ES cell-derived offspring, obtained from breeding germ line chimeras to C57BL/6 or (C57BL/6 × BALB/c)_{F1} females, were considerably smaller than their litter mates (Fig. 1A). Southern Blot analyses with a transgene probe showed that all smaller animals carried the gene trap insertion, whereas none of the normal-size litter mates carried the transgene (data not shown). Both female and male progeny were affected equally, suggesting an insertional mutation with a dominant autosomal pattern of inheritance. The phenotype was obvious already at birth and allowed for identification of transgenic animals at weaning by visual inspection with high fidelity (145 of 153 tested small animals). Transgenic animals had approximately 60% of the body weight of non-transgenic litter mates (Fig. 1B). The phenotype was apparent throughout the postnatal growth period; however, later in adulthood, transgenic animals were almost indistinguishable from wild-type litter mates (data not shown). To address when the growth deficiency is first apparent during development, we analyzed embryos from matings between heterozygous *Gtl2^{lacZ}* males and wild-type females. Embryos between day 12.5 and 18.5 were

weighed and transgenic embryos were identified by staining whole embryos (between day 12.5 and day 14.5) or dissected tissues (between day 16.5 and day 18.5) for β -galactosidase activity. Reduced weight and size were first detected in transgenic fetuses at day 16.5 of embryonic development (data not shown), suggesting that the transgene insertion interferes with the function of a gene required for late fetal and postnatal growth.

To analyze the effect of the transgene in homozygous condition, matings were made between heterozygous *Gtl2^{lacZ}* animals. On a mixed genetic background, wild-type, heterozygous, and homozygous mice were obtained at the expected ratio of 1:2:1, whereas on a predominantly 129/Sv genetic background the viability of heterozygous and homozygous *Gtl2^{lacZ}* mice appeared reduced (Table 1). Homozygous *Gtl2^{lacZ}* mice on both genetic backgrounds were fertile and appeared to have the same size as their heterozygous parents. However, heterozygous offspring obtained from these breedings were either as small as their parents or normal sized, suggesting that the expressivity of the mutation is variable. Subsequent breeding of heterozygous *Gtl2^{lacZ}* mice of both sexes to wild-type mice indicated that the small-size phenotype was influenced by the parental origin of the transgene. When the transgene was transmitted through the male germ line, approximately half of the offspring were obviously small, whereas upon transmission through the female germ line fewer offspring showed a less obvious dwarfism phenotype and were more difficult to distinguish from their normal wild-type litter mates (Table 2). In contrast to the small-size phenotype, transgene expression in het-

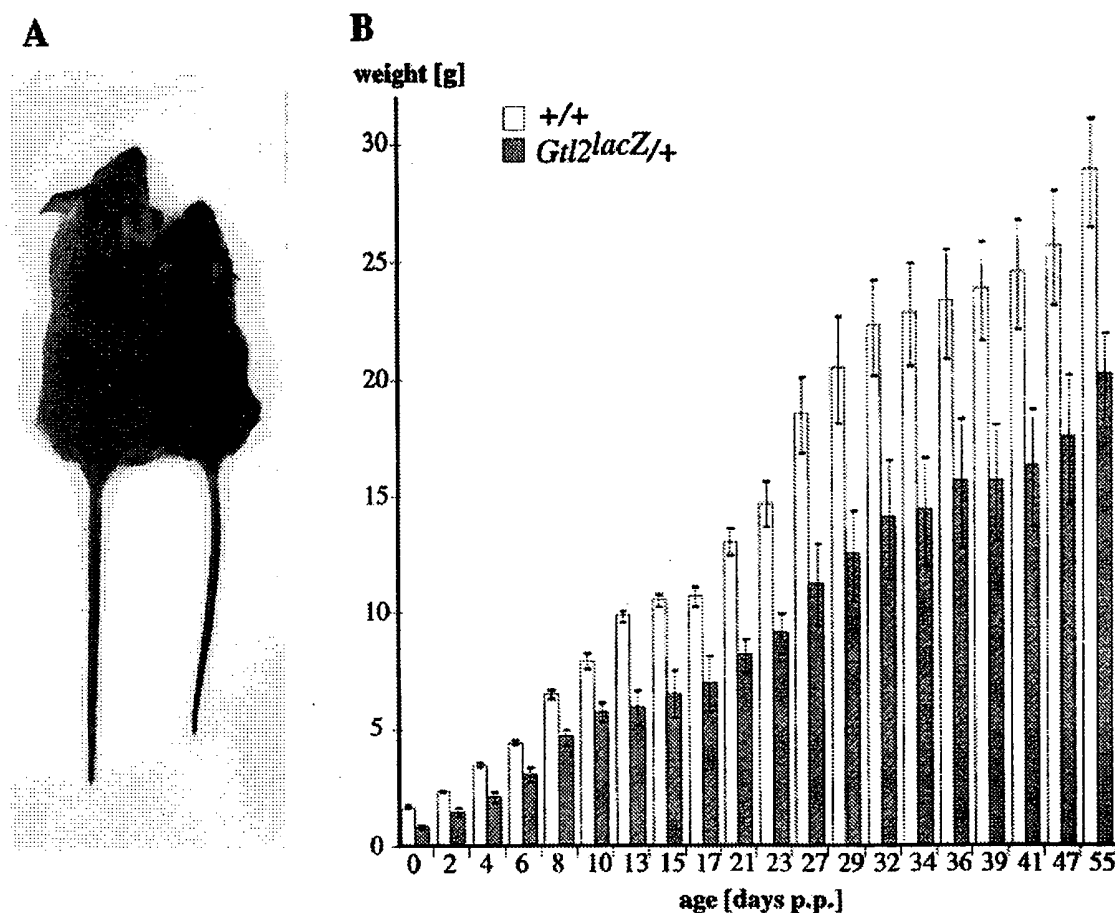


Fig. 1. Phenotype in heterozygous *Gtl2^{lacZ}* transgenic mice after male germ line passage. (A) Wild-type (left) and transgenic (right) litter mates after weaning. (B) Body weight (mean and standard deviation) in wild-type ($n = 5$) and heterozygous ($n = 4$) mice during the postnatal growth period. Transgenic mice were significantly smaller than wild-type litter mates at birth (mean weight 1.72 ± 0.07 vs 0.93 ± 0.11 , $p = 2.4 \times 10^{-6}$) and had a

lower growth rate (0.35 ± 0.032 g/day vs 0.49 ± 0.043 g/day; slopes of growth curves differ significantly $t = 6.6$ with 7 d.o.f.; $p < 0.0003$). Similar weight and growth rate differences between wild-type and heterozygous mice were obtained when small and normal offspring were separated after birth and these groups were raised independently by foster mothers (total of 8 wild-type and 7 heterozygous newborns; data not shown).

Table 1. Transgene inheritance in heterozygous *Gtl2^{lacZ}* matings.

	Genotype of offspring		
	Tg/Tg	Tg/+	+/+
Mixed genetic background	23 (21%)	60 (56%)	25 (23%)
≥75% 129/Sv	16 (14%)	44 (38%)	56 (48%)

erzygous embryos, as assessed by staining for β-galactosidase activity, was not affected by parental legacy.

To test whether the effect of parental origin on the penetrance and severity of the dwarf phenotype is reversible upon subsequent germ line passages of the transgene insertion through the opposite sex, we followed the inheritance of the mutant phenotype through the male and female germ lines over three generations. On a mixed genetic background (predominantly C57BL/6 × BALB/c), the phenotype in heterozygous *Gtl2^{lacZ}* mice was always apparent upon male germ line transmission, regardless whether the transmitting male was a dwarf who had inherited the transgene from the father, or was of normal size and had inherited the transgene from the mother. In contrast, female heterozygous *Gtl2^{lacZ}* mice gave rise only to less obvious small transgenic offspring at a low frequency (Table 3), again irrespective of earlier passages of the transgene through either the male or female germ line. Repeated breeding of *Gtl2^{lacZ}* to 129/Sv mice exclusively through the male germ line resulted in a more severe small-size phenotype. However, upon one passage through the female germ line, no small-size offspring were obtained when the transgene was subsequently transmitted through the male germ line. Thus, strain-specific modifiers appear to influence the effect of the gene trap insertion on the endogenous gene, apparently without modulating the expression of the transgene itself.

The reporter gene integration occurred into one single site into the mouse genome and did not cause rearrangements or deletions detectable by Southern Blot analysis (data not shown). The insertion site was localized to Chr 12 with a flanking probe as described in Materials and Methods and gave the following gene order and distances (in centiMorgans; Table 4):

$$D12Mit4-14.0 \pm 3.5-Gtl2-5.0 \pm 2.1-IgH-V36(D12Nds2)$$

This chromosomal localization excluded that the transgene insertion in *Gtl2^{lacZ}* mice directly affects any of the known imprinted genes or might be a reoccurrence of one of the existing dwarf mutations, since none of these map to Chr 12.

Discussion

We have isolated a transgene-induced dominant insertional mutation on mouse Chr 12 that affects fetal and postnatal growth in a parental origin- and genetic background-dependent manner. The resulting dwarfism phenotype is fully penetrant and strongly expressed after transgene passage through the male germline, whereas penetrance and severity of the small-size phenotype are strongly reduced after passage through the female germ line. This pattern of inheritance of the phenotype is consistent with the idea that the transgene integration has disrupted an endogenous gene(s) that is differentially expressed in the parental alleles, and that expression of the paternal allele contributes more to the functional gene product than expression of the maternal allele. Depending on

Table 2. Influence of parental origin on the penetrance of the *Gtl2^{lacZ}* phenotype.

	Matings			
	Tg/+ male × +/+ female		Tg/+ female × +/+ male	
Phenotype of transgenic mice:	small	normal	small	normal
Number of animals:	30	41	30	217

Table 3. Influence of parental origin and genetic background on penetrance and reversibility of the *Gtl2^{lacZ}* phenotype.

GENERATION	Matings			
	Tg/+ × (C57BL/6 × BALB/c)F ₁		Tg/+ × 129/Sv	
	Sequence of germ line passages ^a	% dwarfs (dwarfs/total)	sequence of germ line passages	% dwarfs (dwarfs/total)
N ₁	M	67 (6/9)	M	40 (6/15)
N ₂	(M).F	12 ^b (11/88)	(M).F	0.5 ^b (6/112)
N ₃	(M.F).F	2.5 ^b (3/123)	(M).M	42 (30/71)
	(M.F).M	39 (53/136)	(M.F).F	0 (0/14) ^d
			(M.F).M	0 (0/30) ^d

^a The germ line passages in a given generation are indicated by M (= male) and F (= female), respectively; germ line passages in previous generations are indicated in parentheses to the left of the most recent germ line passage.

^b Phenotype was less obvious than after male germ line transmission, and small animals were difficult to distinguish from normal litter mates.

^c Five animals were transgenic.

^d Eleven animals were transgenic.

Table 4. Distribution of haplotypes among 100 offspring of a backcross (C57BL/6 × STF)F₁ × C57BL/6.

Markers	Distribution of haplotypes							
D12Mit4	□	■	□	■	□	■	□	■
Gtl2	□	■	■	□	□	■	■	□
D12Nds2 (IgH-V36)	□	■	■	□	■	□	□	■
Total	46	35	9	5	4	1	0	0

For each locus the allele form that has been found is abbreviated as follows: (■) homozygous for C57BL/6, and (□) heterozygous for C57BL/6 and *Mus spretus*.

the parental legacy, the transgene would affect either the more active (paternal) or the less active (maternal) copy, leading to the observed differences in the penetrance and severity of the small-size phenotype. Perturbation of the paternal allele would always decrease the gene product(s) below the threshold level required for normal growth and thus would result in dwarfism. Perturbation of the maternal allele would also affect expression of this gene, but since the maternal allele contributes less to the overall gene product, this reduction might or might not reach the threshold level required for normal growth and thus results in a visible small-size phenotype only in a subset of transgenic mice. It should be pointed out that this scenario assumes partial repression of the maternally derived copy of the gene affected in *Gtl2^{lacZ}* mice, whereas for any of the known imprinted genes only complete repression on one parental chromosome has been observed.

On a mixed genetic background, the transgene effect was reversible upon passage through the germ line of the opposite sex, whereas on a predominantly 129/Sv genetic background, the passage through the female germ line modified the transgene effect, such that upon transmission through the male germ line in the subsequent generation the small-size phenotype remained undetectable. Strong expression of the maternal allele of the endogenous gene on the 129Sv background cannot account for this effect, since passage exclusively through the male germ line on the same background showed the phenotype in all transgenic offspring. Thus, strain-specific modifiers appear to influence penetrance and expressivity of this mutation. These results also suggest that this mutation is not a functional null allele as might be expected for a gene trap insertion that should require integration in a gene to be expressed (Skarnes et al. 1992; Zachgo and Gossler 1994). It appears likely, therefore, that the transgene insertion in *Gtl2* caused a regulatory mutation that can be modulated by the genetic background.

The expression of a transgene can interfere with the transcription of an endogenous gene and lead to a mutant phenotype (Kratohvil et al. 1989, 1993). The *lacZ* gene in *Gtl2^{lacZ}* mice was

expressed from both parental alleles, and expression appeared to be independent of parental legacy. Biallelic expression of the transgene at similar levels could interfere with the transcription of a differentially active maternal and paternal allele of an endogenous gene and could lead to different levels of the endogenous gene product depending on the parental origin of the transgene. Alternatively, parental origin and genetic background could modulate the transcriptional activity of the *lacZ* gene integrated at *Gtl2*, as was observed with other transgene insertions (Hadchouel et al. 1987; Swain et al. 1987; Allen et al. 1990; Chaillet et al. 1995). Modulated transgene expression in turn could interfere with the expression of an endogenous gene in a parental origin- and genetic background-dependent manner, give rise to the observed variable penetrance and expressivity of the phenotype, and mimic differential expression of the parental alleles of an endogenous gene. Since we analyzed *lacZ* expression on the level of the gene product by staining for β -galactosidase activity and not on the transcriptional level, we cannot rule out at present that differences in transcriptional activity, which were not reflected by the β -galactosidase staining, may cause the observed effects.

The chromosomal position of transgene integrations can cause parental origin and genetic background-dependent differences in the methylation state of the transgene. Similarly, a transgene itself can induce methylation differences at the insertion site (Chaillet et al. 1991, 1995; Engler et al. 1991) and can cause variable methylation in the flanking DNA (Reik et al. 1990) in a parental origin-dependent manner. It is conceivable that the transgene insertion in *Gtl2* could induce such methylation differences or modifications of the chromatin structure at the integration site. These could affect the expression of flanking genes in an apparent parental origin-dependent manner and thus mimic a mutation of an imprinted gene.

Gtl2^{lacZ}, like *Adp*, is a transgene insertional mutation on mouse Chr 12 (Watanabe et al. 1994) that shows a dominant modified pattern of inheritance. Maternal or paternal disomies for distal mouse Chr 12 lead to embryonic lethality (Cattanach and Rasberry 1994), and uniparental disomies for the homologous region of human Chr 14 result in defined syndromes (Temple et al. 1991; Wang et al. 1991; Antonarakis et al. 1993; Healey et al. 1994), suggesting that these chromosomal regions in mouse and human contain imprinted genes. The localization of the *Gtl2^{lacZ}* insertion in a region that shows imprinting effects in mice and in humans associated with either maternal or paternal disomy lends further support to the assumption that a gene which is differentially expressed from the two parental alleles might be affected in *Gtl2^{lacZ}* transgenic mice.

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